Secondary-site binding of Glu-plasmin, Lys-plasmin and miniplasmin to fibrin

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Active-site-inhibited plasmin was prepared by inhibition with D-valyl-L-phenylalanyl-L-lysylchloromethane or by bovine pancreatic trypsin inhibitor (Kunitz inhibitor). Active-site-inhibited Glu-plasmin binds far more strongly to fibrin than Glu-plasminogen [native human plasminogen with *N*-terminal glutamic acid (residues 1–790)]. This binding is decreased by α_2 -plasmin inhibitor and tranexamic acid, and is, in the latter case, related to saturation of a strong lysine-binding site. In contrast, α_2 -plasmin inhibitor and tranexamic acid have only weak effects on the binding of Glu-plasminogen to fibrin. This demonstrates that its strong lysine-binding site is of minor importance to its binding to fibrin. Active-site-inhibited Lys-plasmin and Lys-plasminogen (Glu-plasminogen lacking the *N*-terminal residues Glu¹–Lys⁷⁶, Glu¹–Arg⁶⁷ or Glu¹–Lys⁷⁷) display binding to fibrin similar to that of active-site-inhibited Glu-plasmin. In addition, α_2 -plasmin inhibitor or tranexamic acid similarly decrease their binding to fibrin. Glu-plasminogen and active-site-inhibited Glu-plasmin have the same gross conformation, and conversion into their respective Lys- forms produces a similar marked change in conformation [Violand, Sodetz & Castellino (1975) *Arch. Biochem. Biophys.* 170, 300–305]. Our results indicate that this change is not essential to the degree of binding to fibrin or to the effect of α_2 -plasmin inhibitor and tranexamic acid on this binding. The conversion of miniplasminogen (Glu-plasminogen lacking the *N*-terminal residues Glu¹–Val⁴⁴¹) into active-site-inhibited miniplasmin makes no difference to the degree of binding to fibrin, which is similarly decreased by the addition of tranexamic acid and unaffected by α_2 -plasmin inhibitor. Active-site-inhibited Glu-plasmin, Lys-plasmin and miniplasmin have lower fibrin-binding values in a plasma system than in a purified system. Results with miniplasmin(ogen) indicate that plasma proteins other than α_2 -plasmin inhibitor and histidine-rich glycoprotein decrease the binding of plasmin(ogen) to fibrin.

Abbreviations used: KI unit, kallikrein inhibitor unit; NIH unit, National Institutes of Health unit; aprotinin, bovine pancreatic trypsin inhibitor (Kunitz inhibitor); Glu-plasminogen, native human plasminogen with *N*-terminal glutamic acid (residues 1–790); Lys-(Met-, Val-) plasminogen, Glu-plasminogen lacking the *N*-terminal residues Glu¹–Lys⁷⁶ (Glu¹–Arg⁶⁷, Glu¹–Lys⁷⁷); miniplasminogen, Glu-plasminogen lacking the *N*-terminal residues Glu¹–Val⁴⁴¹; K1–5, kringle 1–5 of the plasmin heavy chain defined by Sottrup-Jensen *et al.* (1978). tranexamic acid, *trans*-4-(aminomethyl)cyclohexane-1-carboxylic acid; D-Val-Phe-Lys-CH₂Cl, D-valyl-L-phenylalanyl-L-lysylchloromethane; pNA, 4-nitroanilide; SDS, sodium dodecyl sulphate.

The final step in the activation of the fibrinolytic system is the transformation of plasminogen, which can be described as follows:

All components in the above scheme have been identified in a physiological environment (Thorsen *et al.*, 1981a). Reactions *a* and *c* are catalysed by plasminogen activators and involve the cleavage of

the Arg⁵⁶⁰-Val⁵⁶¹ bond (Robbins *et al.*, 1967; Wiman, 1977; Sottrup-Jensen *et al.*, 1978). This results in the formation of plasmin, composed of a heavy and a light chain connected by two disulphide bridges (Sottrup-Jensen *et al.*, 1978). Reactions *b* and *d* can be catalysed by plasmin and involve the cleavage of the Arg⁶⁷-Met⁶⁸, Lys⁷⁶-Lys⁷⁷ or Lys⁷⁷-Val⁷⁸ bond, associated with the release of the *N*-terminal residues (Claeys *et al.*, 1973; Wiman & Wallén, 1975; Violand & Castellino, 1976). Reaction *a* is regarded as the initial, obligatory event. The rates of the reactions *a-d* depend on a complex interplay between activator, Glu-plasminogen and its derivatives, fibrin and α_2 -plasmin inhibitor (α_2 -antiplasmin). Reaction *b* markedly increases the binding of plasminogen to fibrin (Thorsen, 1975) and α_2 -plasmin inhibitor (Wiman *et al.*, 1979). Binding sites on plasmin(ogen) for fibrin, α_2 -plasmin inhibitor and L-lysine analogues are functionally interdependent (Thorsen, 1975; Wiman & Wallén, 1977; Aoki *et al.*, 1978; Wiman *et al.*, 1978; Christensen *et al.*, 1979; Wiman *et al.*, 1979; Lerch *et al.*, 1980; Thorsen *et al.*, 1981a).

The present study compares the change in secondary-site fibrin binding caused by reaction *a* with those caused by reactions *b-d*. The changes in fibrin binding are studied in the presence of α_2 -plasmin inhibitor, tranexamic acid (an L-lysine analogue) and in a plasma environment. The results are related to those obtained when miniplasminogen (neoplasminogen-Val⁴⁴², K5 light chain), is converted into miniplasmin. Miniplasmin(ogen) lacks important binding sites for α_2 -plasmin inhibitor and L-lysine analogues, but has partially retained binding sites for fibrin (Wiman *et al.*, 1978; Christensen *et al.*, 1979; Thorsen *et al.*, 1981b).

Experimental

Materials

Buffers and chemicals. Tris buffer (*I* 0.15) was Tris/HCl (0.05 mol/litre)/NaCl (0.10 mol/litre), pH adjusted to 7.70 (20°C) with NaOH (1 mol/litre). Tranexamic acid and D-Val-Leu-Lys-pNA (S-2251) were from Kabi Ltd., Stockholm, Sweden. D-Val-Phe-Lys-CH₂Cl (Collen *et al.*, 1980) was used directly as a freeze-dried powder or as a stock solution (1 mmol/litre) in HCl (1 mmol/litre).

Proteins. Aprotinin (Trasylol) and human urokinase have been described (Thorsen & Müllertz, 1974). This also applies to Glu-plasminogen, Lys-plasminogen, miniplasminogen and α_2 -plasmin inhibitor (Thorsen *et al.*, 1981b). Human thrombin (3200 NIH units/mg) was a gift from Dr. W. B. Lawson, New York State Department of Health, Albany, NY, U.S.A., to Dr. I. Clemmensen of our laboratory. Human fibrinogen (Kabi Ltd.) was

purified by removing plasminogen with lysine-Sepharose 4B (Pharmacia, Uppsala, Sweden) (Norén *et al.*, 1975) and by removing Factor XIII with organomercurial-agarose (Affi-gel 501; Bio-Rad Laboratories, Richmond, CA, U.S.A.) (McDonagh *et al.*, 1976). The efficacy of Factor XIII removal was verified by SDS/polyacrylamide-gel electrophoresis, which showed no γ -chain dimerization of fibrin prepared in the presence of CaCl₂ and incubated for 2 h at 37°C. The coagulability of the purified preparation [mean $\pm$ s.e.m. (*n*)] expressed as the ratio [clottable fibrinogen]/[fibrinogen] was 1.0 ± 0.011 (10).

Human plasma. This was obtained from the blood of four normal subjects, freshly drawn with their informed consent, stabilized by 0.1 vol. of trisodium citrate (0.11 mol/litre). Factor XIII was removed by immunoprecipitation at 37°C for 30 min and overnight at 4°C, by using 1 ml of specific rabbit antiserum against Factor XIII (subunit A) in 48 ml of plasma. Clots, prepared in the presence of CaCl₂ and incubated at 37°C for 2 h, could be dissolved within 3 min in acetic acid (0.96 mol/litre)/urea (6.25 mol/litre).

Antibodies. Specific rabbit antiserum against human Factor XIII (subunit A) was from Behringwerke, Marburg/Lahn, Germany. Specific rabbit immunoglobulins against human urokinase have been described (Clemmensen & Christensen, 1976).

Urokinase-Sepharose. This was prepared by coupling urokinase [4×10^4 Ploug units/g of dried CNBr-activated Sepharose 4B (Pharmacia)] according to the manufacturer's instructions.

Methods

Determination of protein concentrations. The concentrations of fibrinogen, clottable fibrinogen, plasmin(ogen) forms and α_2 -plasmin inhibitor were determined as described previously (Thorsen *et al.*, 1981b). The concentration (absorbance) of plasmin in complex with aprotinin was corrected by using an $A_{1\text{cm}, 280}^{1\%}$ value of 8.3 and mol.wt. 6513 (Kassell, 1970).

Radioiodination of plasminogen. This was performed with Enzymobead lactoperoxidase/glucose oxidase (Bio-Rad Laboratories, Richmond, CA U.S.A.) by using ¹²⁵I (Na¹²⁵I, carrier-free, in NaOH solution) from The Radiochemical Centre, Amersham, Bucks., U.K. Plasminogen (200 μ l, 6–16 μ mol/litre) in phosphate buffer (0.2 mol/litre, pH 7.2), 75 μ l of Enzymobead reagent, 1 mCi of Na¹²⁵I (<0.7 nmol) and 75 μ l of β -D-glucose (4125 nmol/litre) were mixed and incubated at room temperature for 15 min. Thereafter 600 μ l of the same plasminogen solution as above was added and unbound iodine and protein were separated by Sephadex G-25 (Pharmacia) gel filtration. The

specific radioactivity ranged between 17 and 60 mCi/ μ mol of plasminogen (calculated for the 200 μ l plasminogen sample). Iodine substitution was less than 0.04 atom of iodine/molecule of plasminogen. Labelled Glu-plasminogen, Lys-plasminogen and miniplasminogen had unaltered electrophoretic mobility in SDS/- and acetic acid/-urea/polyacrylamide-gel electrophoresis. Furthermore, the ratio [concentration (active site)]/[concentration (absorbance)] was unchanged (0.8–1.0) when determined as described by Thorsen *et al.* (1981b).

Preparation of aprotinin-inhibited 125 I-labelled plasmin. This was prepared essentially as described by Sodetz & Castellino (1975) and Summari *et al.* (1975). Tris buffer (I 0.15, 1 ml) containing plasminogen (40–60 μ mol/litre), 125 I-labelled plasminogen (0.3–0.5 μ mol/litre), aprotinin (1.7-fold molar excess over plasminogen) and tranexamic acid (500 μ mol/litre) was mixed with 1 ml of urokinase–Sephacrose in the same buffer and incubated at 37°C. After 150 min the urokinase–Sephacrose was removed by filtration with a Seraclear filter (Technicon Instruments Corporation, Tarrytown, NY, U.S.A.). This was followed by dialysis against Tris buffer (I 0.15) at 4°C for 24 h (aprotinin-inhibited Lys-plasmin) or by gel filtration on Sephadex G-25 (aprotinin-inhibited Glu-plasmin) to remove the tranexamic acid.

Preparation of D-Val-Phe-Lys-CH₂Cl-inhibited 125 I-labelled plasmin. Glu- or Lys-plasminogen or miniplasminogen was activated in Tris buffer (I 0.15) containing plasminogen (4–5 μ mol/litre), 125 I-labelled plasminogen (2–3.5 μ mol/litre), D-Val-Phe-Lys-CH₂Cl (25 mmol/litre for the preparation of inhibited Glu-plasmin, 0.5 mmol/litre for the preparation of inhibited Lys-plasmin and miniplasmin) and urokinase (1.6×10^6 Ploug units/litre) at 37°C, for 60 min (inhibited Glu-plasmin) or 5 min (inhibited Lys-plasmin or miniplasmin). Hereafter, 0.27 vol. of specific rabbit immunoglobulin against urokinase was added to quench the catalytic effect of urokinase. This was followed by gel filtration on Sephadex G-25 or by dialysis against Tris buffer (I 0.15) (4°C, overnight) to remove unbound D-Val-Phe-Lys-CH₂Cl. The dialysed solution was centrifuged to remove the immunoprecipitate.

Polyacrylamide-gel electrophoresis. SDS/polyacrylamide-gel electrophoresis was performed as described by Weber & Osborn (1975), in the presence or absence of urea (6 mol/litre) (Violand & Castellino, 1976). Polyacrylamide-gel electrophoresis in acetic acid/urea at pH 3.2 was performed as previously described (Panyim & Chalkley, 1969; Walther *et al.*, 1975). Electrophoresis was performed in gel slabs (2.7 mm $\times$ 82 mm $\times$ 82 mm) containing acrylamide (73.1 g/litre) and NN'-methylenebisacrylamide (1.9 g/litre). Unreduced samples for acetic acid/urea electro-

phoresis were prepared by diluting the protein solution with 2 vol. of acetic acid (1.75 mol/litre) and urea (9 mol/litre). Reduced samples were prepared by diluting the protein solution with 2 vol. of dithioerythritol (130 mmol/litre) and urea (10 mol/litre). This was followed by incubation for 1 h at 56°C. Acetic acid/urea/polyacrylamide-gel electrophoresis was especially suited to discriminate between Glu-plasminogen and its derivatives (Fig. 1). Electrophoresis of unreduced samples clearly separates Glu-plasminogen from Lys-plasminogen (Walther *et al.*, 1975). Glu- and Lys-plasmin migrate as their respective proenzymes. Electrophoresis of reduced samples separates Glu-plasminogen, Lys-plasminogen, Glu-plasmin heavy chain, Lys-plasmin heavy chain and plasmin light chain (Fig. 1).

Autoradiography of polyacrylamide gels. Gels were shrunk in methanol/glycerol (1:1, by vol.) for 24 h. This was followed by drying. Curix RPL X-Ray Film (Agfa–Gevaert) was used for exposure.

Quantification of 125 I-labelled plasminogen derivatives by polyacrylamide-gel electrophoresis. The bands of labelled plasminogen material were excised from the rest of the gel track and their radioactivity quantified in a gamma well counter. Acetic acid/urea/polyacrylamide-gel electrophoresis of reduced samples yielded bands with the following radioactivities: *a* (Glu-plasminogen); *b* (Lys-plasminogen); *c* (miniplasminogen); *d* (Glu-plasmin heavy chain); *e* (Lys-plasmin heavy chain); *f* (K5); and *g* (plasmin light chain). The sum of the radioactivities of the bands was *t*. The radioactive fraction (=the molar fraction of labelled substance) was calculated as (*a*/*t*) (Glu-plasminogen), (*b*/*t*) (Lys-plasminogen), (*c*/*t*) (miniplasminogen), $\{(d + (d \cdot g)/(d + e))/t\}$ (Glu-plasmin), $\{(e + (e \cdot g)/(d + e))/t\}$ (Lys-plasmin) and $\{(f + g)/t\}$ (miniplasmin). The conversion of 125 I-labelled Glu-plasmin into Lys-plasmin did not change the radioactivity of the heavy chain (S. Müllertz, unpublished work). Acetic acid/urea/polyacrylamide-gel electrophoresis of unreduced samples yielded bands with the following radioactivities: *h* (Glu-plasminogen + Glu-plasmin) and *i* (Lys-plasminogen + Lys-plasmin). The radioactive fraction (=the molar fraction of plasminogen + plasmin) was calculated as $[h/(h + i)]$ (Glu-forms) and $[i/(h + i)]$ (Lys-forms). SDS/polyacrylamide-gel electrophoresis of reduced samples allowed quantification of radioactivities of plasminogen, plasmin heavy-chain, K5 and plasmin light-chain bands. No attempt was made here to distinguish Glu- from Lys-forms. With all forms of electrophoresis, *t* was greater than 0.92 of the total radioactivity in the gel track. The total radioactivity in the gel track, given as a fraction of the radioactivity of the sample applied to the gel, was more than 0.85.

Fig. 1. Acetic acid/urea/polyacrylamide-gel electrophoresis of Glu- and Lys-plasminogen and their respective plasmin forms

Glu-plasminogen: non-reduced (a) and reduced sample (e); Lys-plasminogen: non-reduced (b) and reduced sample (f); Glu-plasmin (prepared from aprotinin-inhibited Glu-plasmin by gel filtration at pH 3): non-reduced (c) and reduced sample (g); Lys-plasmin (prepared from aprotinin-inhibited Lys-plasmin by gel filtration at pH 3): non-reduced (d) and reduced sample (h). The weak bands with greatest mobility in (g) and (h) are the plasmin light chains. The double-banded appearance of the non-reduced samples is presumably due to carbohydrate heterogeneity of the plasmin(ogen) molecules (Walther *et al.*, 1975).

Fibrin binding of labelled plasmin(ogen). This was determined as previously reported (Thorsen *et al.*, 1981b). Clots (vol. 500 μ l) were prepared by adding 50 μ l of thrombin (5×10^4 NIH units/litre) or 50 μ l of the same thrombin solution and 80 μ l of CaCl_2 (50 mmol/litre) to a fibrinogen solution (or plasma) with ^{125}I -labelled ligand. Purified reagents were buffered in Tris, pH 7.70 (20°C) and I 0.15. The fibrin binding [FB(total)], of all labelled ligand preparations was determined as F/T , where F is the fibrin-bound radioactivity, and T is the total radioactivity of the clotting mixture. Active-site-inhibited Glu-plasmin was unstable during the course of the experiments (see the Results section) and minimal values of the fibrin binding of inhibited Glu-plasmin [FB(Glu)] were therefore determined as $(F \cdot x)/(T \cdot y)$. The ratio (radioactivity of Glu-plasminogen + Glu-plasmin)/(total radioactivity of plasminogen material), was determined for the fibrin-bound plasminogen material ($=x$) and for the plasminogen material added to the clotting mixture ($=y$). This was done by acetic acid/urea/polyacrylamide-gel electrophoresis of unreduced samples. Attempts at determining fibrin binding of Glu-plasmin alone, by using acetic acid/urea/polyacrylamide-gel electrophoresis under reducing conditions, were unsuccessful, since

fibrin interfered with the electrophoretic separation.

Results

Preparation conditions and characterization of active-site-inhibited Glu- and Lys-plasmin

The identities of the aprotinin and D-Val-Phe-Lys- CH_2Cl -inhibited plasmin preparations were verified by acetic acid/urea/- and SDS/-polyacrylamide-gel electrophoresis (inhibited Glu-plasmin and miniplasmin are shown in Fig. 2). In addition, the identities of the aprotinin-inhibited plasmin preparations were verified by *N*-terminal amino-acid-sequence analysis (performed by L. Sottrup-Jensen) after separating plasmin from aprotinin by gel filtration on Sephadex G-75 at pH 3 (Suenson *et al.*, 1980). The Glu-plasmin preparation showed the *N*-terminal sequences Glu-Pro (heavy chain) and Val-Val (light chain), and Lys-plasmin showed the *N*-terminal sequences Lys-Val (heavy chain) and Val-Val (light chain). Quantification of the molar fractions of labelled plasminogen derivatives by SDS/- or acetic acid/urea/-polyacrylamide-gel electrophoresis of reduced samples showed preparations of aprotinin-inhibited ^{125}I -labelled Glu-

Fig. 2. Autoradiograph of SDS/polyacrylamide-gel electrophoresis of reduced samples of miniplasmin and Glu-plasmin ligands

(a) D-Val-Phe-Lys-CH₂Cl-inhibited miniplasmin; (b) D-Val-Phe-Lys-CH₂Cl-inhibited Glu-plasmin (the preparation shown is more degraded than those used in the experiments in order to demonstrate the Lys-plasmin contaminant); (c) aprotinin-inhibited Glu-plasmin.

plasmin to contain Glu-plasmin (0.78–0.81), Lys-plasmin (0.12–0.17) and Glu-plasminogen (0.03–0.08). The same values for D-Val-Phe-Lys-CH₂Cl-inhibited ¹²⁵I-labelled Glu-plasmin preparations were 0.69–0.72, 0.14–0.20 and 0.08–0.17. The active-site-inhibited ¹²⁵I-labelled Lys-plasmin and miniplasmin preparations contained more than 0.90 molar fraction of Lys-plasmin and miniplasmin respectively.

Tranexamic acid was present during preparation of aprotinin-inhibited plasmin to increase the rate of activation of Glu-plasminogen and to decrease the non-specific binding of inhibited plasmin to urokinase-Sepharose. Alterations in pH or in the concentration of tranexamic acid in the activation mixture did not decrease the Lys-plasmin contamination of the inhibited Glu-plasmin. A 100-fold molar excess of aprotinin over Glu-plasminogen markedly lowered the rate of activation, but did not improve the preparation. Urokinase-Sepharose and tranexamic acid were avoided in the preparation of D-Val-Phe-Lys-CH₂Cl-inhibited Glu-plasmin as they increased Lys-plasmin contamination to an unacceptable extent.

Accuracy of fibrin-binding measurements

Fibrin binding, measured as FB(total), gave fairly accurate results with the different radiolabelled plasminogens, Lys-plasmin and miniplasmin, since they constituted more than 0.90 of the total radioactivity of the respective preparations. Furthermore, acetic acid/urea/- or SDS/-polyacrylamide-gel electrophoresis of samples of dissolved clots showed

that no degradation of these substances occurred during the course of the experiments. However, FB(total) values of active-site-inhibited Glu-plasmin gave less accurate estimates of its binding, as it constituted only 0.69–0.81 of the radioactivity of these ligand preparations. Furthermore, acetic acid/urea/polyacrylamide-gel electrophoresis of unreduced samples of dissolved clots showed conversion of up to 20% of Glu-plasmin (+Glu-plasminogen) to Lys-plasmin (+Lys-plasminogen) during the course of the experiments. [Proteolytic degradation did not proceed further than the Lys-form (Fig. 3).] Owing to these circumstances, fibrin binding of active-site-inhibited Glu-plasmin was also determined as FB(Glu). This value was an estimate of the lower limit of the fibrin binding of active-site-inhibited Glu-plasmin. The upper limit was FB(total) as there was no evidence of selective adsorption of Glu-plasmin to fibrin. This was documented by the fact that radiolabelled material bound to the separated fibrin had a ratio [Glu-plasmin(+Glu-plasminogen)]/[total plasmin(+total plasminogen)] which did not exceed that in the supernatant after removal of fibrin.

Fibrin-binding of labelled plasminogen derivatives in the simple purified system

Table 1 shows that active-site-inhibited Glu- or Lys-plasmin binds much more strongly to fibrin than does Glu-plasminogen and that they bind to the same extent as, or more strongly than, Lys-plasminogen. FB(total) was the same for aprotinin-inhibited Glu- and Lys-plasmin, indicating that

Fig. 3. Autoradiographs of SDS/- and acetic acid/urea/polyacrylamide-gel electrophoresis of active-site-inhibited Glu-plasmin ligand adsorbed to fibrin

(a) SDS/polyacrylamide-gel electrophoresis of reduced samples of aprotinin-inhibited Glu-plasmin and of (b) D-Val-Phe-Lys-CH₂Cl-inhibited Glu-plasmin. (c) Acetic acid/urea/polyacrylamide-gel electrophoresis of non-reduced samples of aprotinin inhibited Glu-plasmin and of (d) D-Val-Phe-Lys-CH₂Cl-inhibited Glu-plasmin.

Table 1. *Fibrin binding of 125 I-labelled plasminogen derivatives*

In A the clotting mixture contained fibrinogen at $9.2 \mu\text{mol/litre}$ and ligand at $1.2 \mu\text{mol/litre}$; in B concentrations were $5.0 \mu\text{mol}$ and $0.2 \mu\text{mol/litre}$ respectively. The values are given as FB(total), save those in square brackets, which are lower limits given as FB(Glu). For details, see the Experimental and Results sections.

Labelled ligand	Fraction of radioactivity bound to fibrin (mean $\pm$ S.E.M. (n))	
	A	B
Aprotinin-inhibited Glu-plasmin	0.91 ± 0.008 (6) [0.77 ± 0.007]	0.87 ± 0.013 (3) [0.69 ± 0.010]
D-Val-Phe-Lys-CH ₂ Cl-inhibited Glu-plasmin	0.60 ± 0.016 (6) [0.52 ± 0.014]	0.67 ± 0.023 (4) [0.53 ± 0.018]
Glu-plasminogen	0.17 ± 0.009 (6)	0.17 ± 0.024 (7)
Aprotinin-inhibited Lys-plasmin	0.90 ± 0.028 (6)	
D-Val-Phe-Lys-CH ₂ Cl-inhibited Lys-plasmin	0.78 ± 0.006 (3)	0.84 ± 0.015 (2)
Lys-plasminogen	0.71 ± 0.009 (5)	0.70 ± 0.017 (3)
D-Val-Phe-Lys-CH ₂ Cl-inhibited miniplasmin	0.52 ± 0.016 (8)	0.47 ± 0.019 (6)
Miniplasminogen	0.48 ± 0.014 (4)	

they had the same affinity for fibrin. The fibrin binding of the aprotinin-inhibited plasmin forms was higher than that of the D-Val-Phe-Lys-CH₂Cl-inhibited plasmin forms ($P < 0.01$). Conversion of miniplasminogen into D-Val-Phe-Lys-CH₂Cl-inhibited miniplasmin made no difference to the extent to which it bound fibrin.

Aprotinin-inhibited Glu- or Lys-plasmin contained free aprotinin. The molar ratio (free aprotinin)/(aprotinin-plasmin complex) was 0.7. No attempt was made to remove this aprotinin, on account of the instability of the Glu-plasmin preparation, and this excess of aprotinin was shown not to influence the fibrin binding of Lys-plasminogen or D-Val-Phe-Lys-CH₂Cl-inhibited Glu-plasmin.

The urokinase used in the preparation of D-Val-Phe-Lys-CH₂Cl-inhibited plasmin forms was removed by immunoprecipitation in order to avoid plasmin formation in systems containing unlabelled plasminogen. This did not influence the results, since fibrin binding was found to be the same before and after immunoprecipitation in the simple purified system.

A_{280} measurements showed fibrinogen added to the clotting mixtures to be totally recovered as fibrin. This was the case in the presence of each of the examined ligands. However, SDS/polyacrylamide-gel electrophoresis of reduced samples showed some fibrin α -chain degradation in clots containing Lys-plasminogen, miniplasminogen or D-Val-Phe-Lys-CH₂Cl-inhibited Glu-plasmin. The α -chain degradation was presumably caused by trace amounts of free plasmin in the ligand preparations. Addition of aprotinin (in amounts that did not exceed that of free amounts of it in aprotinin-inhibited plasmin) inhibited this degradation and did not alter fibrin binding. Glu-plasminogen and aprotinin-inhibited Glu-plasmin did not produce α -chain degradation.

Fig. 4. Influence of tranexamic acid on the fibrin binding of 125 I-labelled Glu-plasminogen and active-site-inhibited Glu-plasmin

O, Aprotinin-inhibited Glu-plasmin; Δ , D-Val-Phe-Lys-CH₂Cl-inhibited Glu-plasmin; $\blacktriangle$, Glu-plasminogen. Fibrin binding was estimated as FB (total) (—), or as FB(Glu) (---). The clotting mixture contained fibrinogen ($5.0 \mu\text{mol/litre}$) and ligand ($0.2 \mu\text{mol/litre}$). Each point on the Glu-plasminogen curve represents the mean of four to six determinations. All other points represent the mean of three determinations.

Fibrin-binding of labelled plasminogen derivatives in the presence of tranexamic acid or α_2 -plasmin inhibitor and in plasma clots

Figs. 4 and 5 show that micromolar concentrations of tranexamic acid decreased the fibrin binding of Lys-plasminogen and inhibited Glu- and Lys-plasmin, but had no effect on Glu-plasminogen. The decrease in fibrin binding of Glu-plasminogen was insignificant ($P < 0.20$) at all concentrations of tranexamic acid ($10\text{--}330\text{ }\mu\text{mol/litre}$) in Fig. 4. In Fig. 5 the decrease was significant ($P < 0.001$) at 1 and 10 mmol/litre . It is unlikely that the weak effect of tranexamic acid on fibrin binding was related to non-specific inclusion of the Glu-plasminogen–tranexamic acid complex in the fibrin network. This is substantiated by the fact that the fibrin binding of a variant of K1+2+3 is the same as that of Glu-plasminogen and can be decreased from 0.17 to 0.065 by tranexamic acid at $10\text{ }\mu\text{mol/litre}$, and further to 0.040 by tranexamic acid at $100\text{ }\mu\text{mol/litre}$

Fig. 5. Influence of tranexamic acid on the fibrin binding of ^{125}I -labelled Glu- and Lys-plasminogen and their respective inhibited plasmin forms

○, Aprotinin-inhibited Glu-plasmin; ●, aprotinin-inhibited Lys-plasmin; □, D-Val-Phe-Lys-CH₂Cl-inhibited Lys-plasmin; ▲, Glu-plasminogen; ■, Lys-plasminogen. Fibrin binding was estimated as FB(total). The clotting mixture contained fibrinogen ($9.2\text{ }\mu\text{mol/litre}$) and ligand ($1.2\text{ }\mu\text{mol/litre}$). The points corresponding to tranexamic acid concentrations of 0, 10^3 and $10^4\text{ }\mu\text{mol/litre}$ on the Glu-plasminogen curve represent the mean of five or six determinations. All other points represent the mean of at least two determinations.

(Thorsen *et al.*, 1981b). Tranexamic acid decreased the fibrin binding of D-Val-Phe-Lys-CH₂Cl-inhibited miniplasmin to the same extent as previously reported for miniplasminogen (Thorsen *et al.*, 1981b). The concentration of the tranexamic acid that decreased fibrin binding of ligand by 50% was $110\text{ }\mu\text{mol/litre}$ for inhibited miniplasmin and $80\text{ }\mu\text{mol/litre}$ for miniplasminogen (fibrinogen, $9.2\text{ }\mu\text{mol/litre}$; ligand, $1.2\text{ }\mu\text{mol/litre}$).

α_2 -Plasmin inhibitor decreased the fibrin binding of Lys-plasminogen, and D-Val-Phe-Lys-CH₂Cl-inhibited Glu- and Lys-plasmin (Fig. 6), but its effect on labelled Glu-plasminogen was weak (at concentrations up to $10.9\text{ }\mu\text{mol/litre}$). It had no effect whatever on labelled miniplasminogen and D-Val-Phe-Lys-CH₂Cl-inhibited miniplasmin (results not shown). Indirect binding of plasminogen derivatives to fibrin through α_2 -plasmin inhibitor was avoided by using a Factor XIII-free assay system (Aoki & Sakata, 1980; Sakata & Aoki, 1980).

The fibrin binding of Lys-plasminogen, D-Val-Phe-Lys-CH₂Cl-inhibited Glu-plasmin, Lys-plasmin and miniplasmin was 1.8–2.8 times higher in fibrin clots than in plasma clots (Table 2). Addition of the

Fig. 6. Influence of α_2 -plasmin inhibitor on the fibrin binding of ^{125}I -labelled Glu- and Lys-plasminogen and their respective active-site-inhibited plasmin forms
△, D-Val-Phe-Lys-CH₂Cl-inhibited Glu-plasmin; □, D-Val-Phe-Lys-CH₂Cl-inhibited Lys-plasmin; ▲, Glu-plasminogen; ■, Lys-plasminogen. Fibrin binding was estimated as FB(total) (—), or as FB(Glu) (---). The clotting mixture contained fibrinogen ($5.0\text{ }\mu\text{mol/litre}$) and ligand ($0.2\text{ }\mu\text{mol/litre}$). Each point represents the mean of at least two determinations.

Table 2. Fibrin binding of ^{125}I -labelled plasminogen derivatives in clotted plasma (A) clotted fibrinogen with Glu-plasminogen and α_2 -plasmin inhibitor (B), and in clotted fibrinogen alone (C)

The clotting mixtures all contained fibrinogen (5.0 $\mu\text{mol/litre}$), ligand (0.2 $\mu\text{mol/litre}$) and CaCl_2 (8 mmol/litre). A and B also contained Glu-plasminogen (1.7 $\mu\text{mol/litre}$) and α_2 -antiplasmin (0.9 $\mu\text{mol/litre}$). In A, 0.63 of the volume of the reaction mixtures was plasma. All values are given as FB(total), save those in square brackets, which are lower limits given as FB(Glu).

Radiolabelled ligand	Fraction of radioactivity bound to fibrin (mean, $n = 2$)		
	A	B	C
D-Val-Phe-Lys- CH_2Cl -inhibited Glu-plasmin	0.39 [0.34]	0.63 [0.55]	0.72 [0.63]
D-Val-Phe-Lys- CH_2Cl -inhibited Lys-plasmin	0.35	0.64	0.85
D-Val-Phe-Lys- CH_2Cl -inhibited miniplasmin	0.17	0.48	0.47
Lys-plasminogen	0.38	0.68	0.83

same amounts of Glu-plasminogen and α_2 -plasmin inhibitor in the fibrin clots as were present in the plasma clots did not decrease the fibrin binding to the values found for plasma clots. These values were the same for Lys-plasminogen and active-site-inhibited Glu- and Lys-plasmin. The experiments were performed in the presence of CaCl_2 (8 mmol/litre), which did not alter the binding of plasminogen derivatives to fibrin (compare with Table 1).

Discussion

Conversion of Glu-plasminogen to active-site-inhibited Glu- or Lys-plasmin or to Lys-plasminogen results in a marked increase in fibrin binding (Table 1). This corresponds to a 5–50-fold decrease in the apparent dissociation constant (Table 3). Glu- and Lys-plasmin(ogen) contain a number of sites, denoted 'lysine-binding sites', on the K1–4 parts of the molecules, that bind lysine analogues (Markus *et al.*, 1978, 1979, 1981). One of these sites on K1–3 has a particularly strong affinity. This site has been ascribed major importance in the binding of plasmin(ogen) to fibrin and α_2 -plasmin inhibitor (Wiman & Wallén, 1977; Wiman & Collen, 1978; Wiman *et al.*, 1979). However, the observation that tranexamic acid at concentrations below 330 $\mu\text{mol/litre}$ has no effect on the fibrin binding of Glu-plasminogen (Fig. 4) indicates that the strong lysine-binding site is not of major importance for the binding of Glu-plasminogen to fibrin. Micromolar concentrations of tranexamic acid radically decrease the fibrin binding of inhibited Glu- and Lys-plasmin and Lys-plasminogen (Figs. 4 and 5). The calculated dissociation constants in Table 3 indicate that this is related to saturation of a strong lysine-binding site. The potent effect of tranexamic acid after formation of Glu- or Lys-plasmin or Lys-plasminogen may be explained as follows. On conversion of Glu-plasminogen, the strong lysine-binding site of the proenzyme adopts a favourable conformation so as

also to bind fibrin. Alternatively, a hidden strong lysine-binding site is exposed that binds fibrin. In both cases tranexamic acid decreases fibrin binding by a competitive effect. There is, however, also the possibility that tranexamic acid may act as an allosteric effector, making a separate fibrin-binding site in the Glu-plasminogen derivative inaccessible to fibrin.

The weak effect of α_2 -plasmin inhibitor on the fibrin binding of Glu-plasminogen (Fig. 6) is compatible with the relatively low affinity between Glu-plasminogen and the inhibitor that was reported by Wiman *et al.* (1979). The effect of micromolar concentrations of α_2 -plasmin inhibitor on the fibrin binding of D-Val-Phe-Lys- CH_2Cl -inhibited Glu- or Lys-plasmin or on Lys-plasminogen (Fig. 6, Table 3) indicates that secondary-site α_2 -plasmin inhibitor affinity increases when Glu-plasminogen is converted into each of these derivatives.

Sedimentation-velocity experiments have shown Glu-plasminogen and aprotinin-inhibited Glu-plasmin to have the same gross conformation. Conversion of Glu-plasminogen and inhibited Glu-plasmin to their respective Lys- forms produce a similar marked change in conformation (Violand *et al.*, 1975). This change resembles that seen on complex-formation with L-lysine analogues (Violand *et al.*, 1975), and seems mainly to involve the K1–5 part of the plasminogen molecule (Gonzales-Gronow *et al.*, 1977). Our results indicate that this change in conformation is not essential to a high degree of fibrin binding or to the effect of α_2 -antiplasmin or tranexamic acid on this binding.

Aprotinin-inhibited Glu- or Lys-plasmin display higher fibrin binding than do the D-Val-Phe-Lys- CH_2Cl -inhibited Glu- or Lys- forms. This difference could be due to side reactions of the chloromethane inhibitor (Shaw, 1975) causing modification of ligand residues involved in secondary-site fibrin binding.

Cederholm-Williams & Swain (1979), found

Table 3. Apparent dissociation constants (K) for the labelled plasminogen derivatives and fibrin monomer, α_2 -antiplasmin or tranexamic acid

$K_{(\text{fibrin})} = K_f = c_f \cdot [(1/\text{FB}) - 1]$ was calculated from the data in Table 1; $K(\alpha_2\text{-antiplasmin/tranexamic acid}) = [c_1 \cdot K_f] / [(c_f/\text{FB}) - (K_f + c_f)]$ was calculated from the data in Figs. 4–6 and from miniplasmin(ogen) data referred to in the Results section. $\text{FB} = \text{FB}(\text{total})$ or $\text{FB}(\text{Glu})$; c_f = fibrinogen concentration in the clotting mixture (= free + bound fibrin monomer concentration); c_1 = free + bound α_2 -antiplasmin tranexamic acid concentration. The $K_{(\alpha_2\text{-antiplasmin})}$ was calculated for $c_1 = 3 \mu\text{mol/litre}$; $K_{(\text{tranexamic acid})}$ was calculated for c_1 = concentration of tranexamic acid that decreases FB by 50%. It was assumed that most of c_f and c_1 represented free substance, that α_2 -antiplasmin tranexamic acid did not bind to fibrin and that single association reactions were involved. The K values are derived from $\text{FB}(\text{total})$, save those in square brackets, which are derived from $\text{FB}(\text{Glu})$. In A, the clotting mixture contained fibrinogen ($9.2 \mu\text{mol/litre}$), ligand ($1.2 \mu\text{mol/litre}$). In B, it contained fibrinogen ($5.0 \mu\text{mol/litre}$) and ligand ($0.2 \mu\text{mol/litre}$). *, K values from Wiman *et al.* (1979); **, K values from Markus *et al.* (1979).

Labelled ligand	Dissociation constant (K) ($\mu\text{mol/litre}$)				
	Fibrin		α_2 -Antiplasmin	Tranexamic acid	
	A	B	B	A	B
Aprotinin-inhibited Glu-plasmin	0.90 [2.7]	0.75 [2.2]		12	25 [66]
D-Val-Phe-Lys-CH ₂ Cl-inhibited Glu-plasmin	6.1 [8.5]	2.5 [4.4]	2.8 [3.6]		17 [19]
Glu-plasminogen	45	24	(4.0)*	(1.1, 750)**	
Aprotinin-inhibited Lys-plasmin	1.0			9.1	
D-Val-Phe-Lys-CH ₂ Cl-inhibited Lys-plasmin	2.6	1.0	0.58	11	
Lys-plasminogen	3.8	2.1	1.3 (0.63)*	7.3 (2.2, 36, 1035)**	
D-Val-Phe-Lys-CH ₂ Cl-inhibited miniplasmin	8.5	5.6		53	
Miniplasminogen	10			41	

p-nitrophenyl *p*'-guanidinobenzoate-inhibited Lys-plasmin to bind strongly to fibrin. However, their plasmin may have been structurally altered, since it had been exposed to pH 2.5 during preparation. Acid treatment changes the physico-chemical and functional properties of Glu-plasminogen (Alkjaersig, 1964; Thorsen, 1977; Claeys & Collen, 1978) and markedly increases its fibrin binding (S. Thorsen, unpublished work). Their clotting mixtures contained nanomolar concentrations of fibrinogen and ligand, and fibrin was enmeshed in crushed glass. In our hands these conditions introduce a significant error due to the adsorption of proteins to glass. This alone may explain why their dissociation constants for 'Lys'-plasmin(ogen) and fibrin are approx. 100 times lower than those in Table 3.

The previously reported results on the fibrin binding of miniplasminogen (Thorsen *et al.*, 1981b) are extended with the observation that conversion of miniplasminogen into D-Val-Phe-Lys-CH₂Cl-inhibited miniplasmin makes no difference to the degree of fibrin binding (Table 1) or to the effect of α_2 -antiplasmin or tranexamic acid on this binding. For further discussion on this subject, see Thorsen *et al.* (1981b).

Tables 1 and 3 (columns headed B) show that the ligands associate more strongly to fibrin in the reaction mixtures containing low concentrations of

fibrinogen and ligand. Separate experiments have shown this to be exclusively correlated with the fibrinogen concentration (Thorsen, 1975; S. Thorsen, unpublished work). The kinetics of the fibrin binding of ligands may therefore be more complex than assumed in Table 3 and may involve a heterogeneous population of fibrin monomers at different stages of polymerization.

Active-site-inhibited Glu-plasmin, Lys-plasmin and miniplasmin have low fibrin-binding values in plasma systems as compared with purified systems (Table 2). Similar results have previously been reported for their proenzymes (Rákóczi *et al.*, 1978; Lijnen *et al.*, 1980; Thorsen *et al.*, 1981b). The low fibrin binding in the plasma system of Glu-plasmin(ogen) and Lys-plasmin(ogen) can to a major extent be explained by a combined effect of α_2 -plasmin inhibitor and histidine-rich glycoprotein (Lijnen *et al.*, 1980). However, the low fibrin binding in a plasma system of miniplasminogen and inhibited miniplasmin cannot be explained by a combined effect of α_2 -plasmin inhibitor and the histidine-rich glycoprotein, since miniplasmin(ogen) lacks important binding sites for these two proteins (Wiman *et al.*, 1978, 1979; Christensen *et al.*, 1979; Lijnen *et al.*, 1980). This finding indicates that additional plasma proteins might well be involved in the low fibrin binding of plasmin(ogen) in plasma.

In conclusion, the results of the present study and that of Thorsen *et al.* (1981b) indicate that binding sites for fibrin, α_2 -antiplasmin and L-lysine analogues are interdependent, but may well not be identical.

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