Sequence of formation of molecular forms of plasminogen and plasmin-inhibitor complexes in plasma activated by urokinase or tissue-type plasminogen activator

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The pathway of plasminogen transformation was studied in plasma, particularly in relation to fibrin formation and the subsequent stimulation of plasminogen activation. Plasminogen was activated by urokinase (low fibrin-affinity) or tissue-type plasminogen activator (high fibrin-affinity). Formation of ^{125}I -labelled free and inhibitor-bound plasminogen derivatives was quantified after their separation by acetic acid/urea/polyacrylamide-gel electrophoresis. In plasma activator converted Glu-plasminogen (residues 1–790) into Glu-plasmin, which was complexed to α_2 -plasmin inhibitor. When this inhibitor was saturated, Glu-plasmin was autocatalytically converted into Lys-plasmin (residues 77–790). No plasmin-catalysed Lys-plasminogen formation was observed. Upon fibrin formation, activation initially followed the same Glu-plasminogen-into-Glu-plasmin conversion pathway, and stimulation of plasminogen activation was only observed with tissue-type plasminogen activator. In agreement with the emergence of novel effector function, on early plasmin cleavage of fibrin [Suenson, Lützen & Thorsen (1984) *Eur. J. Biochem.* **140**, 513–522] the fibrin-binding of Glu-plasminogen increased when solid-phase fibrin showed evident signs of degradation. This was associated with the formation of considerable amounts of the more easily activatable Lys-plasminogen, most of which was fibrin-bound. At the same time the rate of plasmin formation with urokinase increased over that in unclotted plasma and the rate of plasmin formation with tissue-type plasminogen activator accelerated. Altogether these processes favoured enhanced fibrin degradation. The rates of Lys-plasminogen and plasmin formation abruptly decreased after lysis of fibrin, probably owing to a compromised effector function on further fibrin degradation.

There are two immunologically distinct well-characterized types of plasminogen activator responsible for conversion of plasminogen into plasmin, urokinase and tissue-type activator (Kok, 1979; Rijken *et al.*, 1981; Günzler *et al.*, 1982; Pennica *et al.*, 1983). Both are present in many tissues, extravascular body fluids and blood (Collen, 1980; Rijken *et al.*, 1981; Holmberg *et al.*, 1982; Wun *et al.*, 1982). The tissue-type plasmin-

ogen activator is considered to be the physiologically most important activator in blood as regards intravascular fibrin re-solution.

Fibrin accelerates its own re-solution by serving as a cofactor for activation of plasminogen (Müllertz, 1956). The tissue-type plasminogen activator binds strongly to fibrin, and the catalytic effect of this activator is especially dependent on its cofactor. This has been shown both in qualitative studies (Camiolo *et al.*, 1971; Thorsen *et al.*, 1972; Wallén, 1977; Matsuo *et al.*, 1981; Mattson *et al.*, 1981) and in more-recent kinetic studies (Hoylaerts *et al.*, 1982; Rijken *et al.*, 1982; Rånby, 1982).

The significance and the mechanism of regulation of the reactions involved in fibrinolysis remain

Abbreviations used: NIH units, National Institutes of Health unit; Glu-plasminogen (plasmin), human plasminogen (plasmin) with *N*-terminal glutamic acid (residues 1–790); Lys/Met/Val-plasminogen (plasmin), human plasminogen (plasmin) fragments with *N*-terminal lysine/methionine/valine (residues 77–790, 68–790 and 78–790).

to be fully elucidated in a physiological environment. In complementary work to the present study we have identified molecular forms of plasminogen and of plasmin complexed to α_2 -plasmin inhibitor and to α_2 -macroglobulin during activation of plasminogen in plasma and clotted plasma (Müllertz *et al.*, 1984). The object of the present study was to quantify these components and to establish the sequence of their formation during activation by urokinase or tissue-type plasminogen activator. Preliminary data on part of this work have been presented (Thorsen & Müllertz, 1979).

Experimental

Materials

Tris buffer (10.15), pH 7.70 (20°C), gelatin/Tris buffer (10.15), pH 7.70 (20°C), ^{125}I -labelled human Glu- and Lys-plasminogen, bovine thrombin (95 NIH units/mg) and human urokinase (7700 Ploug units/mg) have been described previously (Thorsen, 1975; Thorsen *et al.*, 1981). Pig tissue plasminogen activator was prepared as described previously (Kok & Astrup, 1969). Human dichain melanoma-cell plasminogen activator (Rijken & Collen, 1981) was a gift from Dr. Collen, University of Leuven, Leuven, Belgium. Human plasma deficient in Factor XIII was prepared by immunoprecipitation of Factor XIII in pooled freshly drawn plasma (four to six normal subjects), stabilized by 0.1 vol. of trisodium citrate (110 mM) (Suenson & Thorsen, 1981).

Methods

Protein concentrations. The concentration of purified plasminogen and the plasma concentrations of Glu-plasminogen and inhibitors were determined as described (Müllertz *et al.*, 1984). The concentration of clottable fibrinogen in plasma was determined spectrophotometrically at 280 nm. A mixture of 0.5 ml of plasma and 8 ml of 6-aminohexanoic acid (31 mM)/ Na_2HPO_4 (3.54 mM)/ NaH_2PO_4 (14.4 mM)/NaCl (125 mM), pH 6.1, was clotted with 1 ml of bovine thrombin (10^5 NIH units/l)/ CaCl_2 (24 mM). After 10 min at 37°C the fibrin was removed and processed as previously reported (Thorsen, 1975). Molar concentrations of urokinase were calculated by using $1 \text{ mol} \equiv 5.6 \times 10^{12}$ Ploug units (Christensen *et al.*, 1982). Concentrations (molar) of melanoma-cell plasminogen activator were calculated by using its mass concentration and $M_r = 72000$ (Rijken & Collen, 1981). Concentrations (molar) of pig tissue plasminogen activator were calculated by using $1 \text{ mol} \equiv 1.2 \times 10^{14}$ Albrechtsen & Astrup units (P. Kok, unpublished work).

Polyacrylamide-gel electrophoresis. This was per-

formed in acetic acid/urea at pH 3.2 (non-reduced samples) or in sodium dodecyl sulphate (reduced samples) as described in the preceding paper (Müllertz *et al.*, 1984).

Quantification of the transformation of ^{125}I -labelled Glu-plasminogen in plasma and clotted plasma. Plasma containing ^{125}I -labelled Glu-plasminogen was mixed with activator in gelatin/Tris buffer, or with a mixture of activator and thrombin in the same buffer (clotted plasma). Identical reaction mixtures (200 μl) were incubated at 37°C and the reaction in these was quenched at given times by addition of 2 vol. of acetic acid (1.75 M)/urea (9 M). The mixtures were stored at 4°C and subjected to acetic acid/urea/polyacrylamide-gel electrophoresis within 2 h (short preincubation) (Müllertz *et al.*, 1984). This was followed by autoradiography and quantification of free and inhibitor-bound plasminogen derivatives in excised gel sections (Suenson & Thorsen, 1981). The amount of labelled Glu-plasminogen at zero time, expressed as a fraction of the total amount of plasminogen, ranged between 0.02 and 0.19. The final concentration of thrombin in clotted plasma was 10^4 NIH units/l.

Quantification of the degradation of fibrinogen in plasma. Plasma samples with added activator (volume 500 μl) were prepared and incubated as described above. At given times A_{280} of clottable fibrinogen was determined. The rate of fibrinogen degradation was expressed as the time when the A_{280} of clottable fibrinogen was decreased to 0.5 of its original value. This was calculated by interpolation on a curve of A_{280} as a function of time.

Quantification of fibrin-binding of labelled plasminogen and labelled plasmin- α_2 -plasmin inhibitor complex. Clotted plasma samples (500 μl) were prepared and incubated as described above. For each single determination two identical clots (A and B) were prepared. After a given time the fibrin threads in A were wound up on to a glass rod at room temperature during 1 min. The fibrin was squeezed free of liquid, washed briefly in ice-cold Tris buffer, dried by blotting on a piece of filter paper and solubilized in 1 ml of acetic acid (1.25 M)/urea (6 M). Then the reaction was quenched in the residual liquid phase of A and in the total of B by addition of 2 vol. of acetic acid (1.75 M)/urea (9 M). This was followed by acetic acid/urea/polyacrylamide-gel electrophoresis and quantification of plasminogen and plasmin-inhibitor complexes in each of the three acidified samples as described previously (Suenson & Thorsen, 1981). The total amount of a plasminogen derivative (B) did not differ significantly from the sum of the amounts of the same derivative in the liquid phase and in the fibrin (A). The recovery of fibrin material was determined by measuring A_{280} after dilution of the

acetic acid/urea-solubilized fibrin with 10 vol. of NaOH (1 M) (Thorsen, 1975).

Results

Sequence of transformation of Glu-plasminogen effected by urokinase

Electrophoretic pattern. Autoradiographs of polyacrylamide-slab-gel electrophoretograms showing the transformation of Glu-plasminogen in urokinase-activated plasma and clotted plasma are shown in Fig. 1. The radioactive bands (*a-f*, defined in the legend to Fig. 1) were identified in our preceding work (Müllertz *et al.*, 1984). Free Glu- and Lys-plasmin migrated as their analogous plasminogen forms. They constituted a negligible fraction of plasmin + plasminogen (Müllertz *et al.*, 1984).

Transformation of plasminogen in plasma. The components detected in Fig. 1 were quantified. In plasma (Fig. 2, $\diamond$, $\square$, $\circ$, $\triangle$ and ∇ symbols) the decrease in Glu-plasminogen concentration with time was associated with a corresponding increase in the concentration of inhibitor-bound plasmin. The amount of plasmin formed approached a constant level, which was about 0.4 of the initial Glu-plasminogen concentration. The fraction of plasmin bound to α_2 -plasmin inhibitor decreased from 0.93 to 0.77 during the experiment. The rest

was bound to α_2 -macroglobulin. The fraction of α_2 -plasmin inhibitor bound to plasmin attained a maximal value of 0.58. Glu-plasmin was the dominant form in the plasmin- α_2 -plasmin inhibitor complex (Fig. 1), constituting 0.7 of the total amount of plasmin bound to this inhibitor at 6 ks. Lys-plasminogen was not detectable.

Addition of large amount of urokinase to activated plasma at a time when the reaction rates approached zero (Table 1) led to conversion of residual plasminogen into plasmin, and to a significant increase in the amount of plasmin complexed both to α_2 -plasmin inhibitor and to α_2 -macroglobulin.

Apart from quantitative differences in reaction rates, similar results were obtained when the urokinase concentration of the experiment shown in Figs. 1 and 2 was increased or decreased 2-fold.

Transformation of plasminogen in clotted plasma. In clotted plasma (Fig. 2, $\diamond$, $\blacksquare$, $\bullet$, $\blacktriangle$ and $\blacktriangledown$ symbols) marked Lys-plasminogen formation was observed, as opposed to the result obtained in plasma. After a lag phase its concentration increased. It reached a maximum soon after lysis of fibrin and then decreased. The values observed were the resultant of the ratio of the rate of Lys-plasminogen formation to the rate of transformation of this component into Lys-plasmin, the ratio changing from >1 through 1 to <1 .

Fig. 1. Autoradiographs of gel slabs after acetic acid/urea/polyacrylamide-gel electrophoresis, showing the transformation of Glu-plasminogen in plasma and clotted plasma activated by urokinase

Samples of plasma and clotted plasma with added ^{125}I -labelled Glu-plasminogen were activated by urokinase at 37°C . At set times of incubation the reaction in these samples was quenched and samples were subjected to acetic acid/urea/polyacrylamide-gel electrophoresis followed by autoradiography. The radioactive bands have been identified in preceding work (Müllertz *et al.*, 1984). They are plasmin- α_2 -macroglobulin complex (*a*), Glu-plasmin- α_2 -plasmin inhibitor complex (*b*), Lys-plasmin- α_2 -plasmin inhibitor complex (*c*), cathodic Lys-plasmin- α_2 -plasmin inhibitor complex (*d*), Glu-plasminogen (*e*) and Lys-plasminogen (*f*). The values above each gel track indicate the reaction time in ks. Reaction mixtures contained initially urokinase (2.9 nM), Glu-plasminogen (2.4 μM), clottable fibrinogen (6.4 μM), α_2 -plasmin inhibitor (1.2 μM) and α_2 -macroglobulin (1.9 μM). The plasma volume fraction was 0.85.

Fig. 2. Concentration of plasminogen and plasmin-inhibitor complexes as a function of time in plasma and clotted plasma activated by urokinase

Experimental conditions and initial component concentrations in reaction mixtures are those indicated for Fig. 1. After separation by acetic acid/urea/polyacrylamide-gel electrophoresis, the concentrations of plasminogen and plasmin-inhibitor complexes in incubated plasma and clotted plasma were determined by quantification of radioactive bands in excised gel sections as described in the Experimental section. Glu-plasminogen in plasma ($\diamond$) and clotted plasma ($\blacklozenge$), Lys-plasminogen in plasma ($\square$) and clotted plasma ($\blacksquare$), plasmin complexed to α_2 -plasmin inhibitor + plasmin complexed to α_2 -macroglobulin in plasma ($\circ$) and clotted plasma ($\bullet$), plasmin complexed to α_2 -plasmin inhibitor in plasma ($\triangle$) and clotted plasma ($\blacktriangle$), and plasmin complexed to α_2 -macroglobulin in plasma (∇) and clotted plasma ($\blacktriangledown$) were investigated. The broken curve (----) indicates the expected formation of inhibitor-bound plasmin in plasma if it had followed first-order kinetics. This curve was constructed by using a first-order rate constant $= v_0/[Pg_0]$, where v_0 is the initial rate of formation of inhibitor-bound plasmin in plasma and $[Pg_0]$ is the initial concentration of Glu-plasminogen. The arrows indicate the time when A_{280} of clottable fibrinogen in plasma decreased to 0.5 of its original value ($\downarrow$) and the time when the fibrin in the clotted plasma lysed ($\downarrow$). Each point on the curves is the mean of three determinations with S.E.M. $\leq 0.004 \mu M$.

The rate of formation of inhibitor-bound plasmin in plasma and clotted plasma was the same at early stages of the activation process (Fig. 2a). Later, when Lys-plasminogen was formed in clotted plasma, the rate of plasmin formation increased over that in plasma ($P < 0.05$ at a reaction time > 0.84 ks; Student's t test). After lysis of fibrin (at about 3 ks) the rate of formation of inhibitor-bound plasmin abruptly decreased. A larger fraction of plasminogen was converted into inhibitor-bound plasmin in clotted plasma than in plasma (0.7 versus 0.4). A smaller fraction of this plasmin was eventually bound to α_2 -plasmin inhibitor (0.58 versus 0.77), a larger fraction was bound to α_2 -macroglobulin, and a larger fraction of α_2 -plasmin inhibitor was complexed to plasmin (0.76 versus 0.58).

Initially in clotted plasma Glu-plasmin dominated in the plasmin- α_2 -plasmin inhibitor complex (Fig. 1). When Lys-plasminogen was formed, Lys-plasmin- α_2 -plasmin inhibitor complex became more dominant. Eventually all complexed plasmin was converted into its Lys-form. A large fraction of the Lys-plasmin- α_2 -plasmin inhibitor complex was partly transformed into a more cathodic and presumably more degraded form. This occurred at about 1.8 ks, when reactive α_2 -plasmin inhibitor was still present. Plasmin complexed to α_2 -macroglobulin was mainly in its Lys-form (Müllertz *et al.*, 1984).

Addition of a large amount of urokinase to activated clotted plasma, at a time when the reaction rates approached zero, led to a significant increase in the amount of plasmin bound to α_2 -macroglobulin only, indicating that more reactive plasminogen and α_2 -macroglobulin were present, while α_2 -plasmin inhibitor was saturated with plasmin.

The fibrin in clotted plasma lysed within 1.25 ks (Fig. 2). This occurred before A_{280} of clottable fibrinogen in plasma decreased to 0.5 of its original value (at 4.55 ks). The plasma became unclottable between 6 and 12 ks.

Apart from quantitative differences in reaction rates, similar results were obtained when the concentration of urokinase was increased or decreased 2-fold in clotted plasma of the experiment shown in Figs. 1 and 2. No transformation of Glu-plasminogen took place in clotted plasma without added activator when it was incubated at $37^\circ C$ for 24 h.

Influence of Factor XIII. Plasma made deficient in Factor XIII was used in the present study. This made it possible to solubilize clotted plasma and study the transformation of Glu-plasminogen before clot lysis. In the preceding paper (Müllertz *et al.*, 1984) free and inhibitor-bound plasminogen derivatives were isolated and identified in an urokinase-activated plasma system containing Factor XIII. A comparison of Table 1 of that paper

Table 1. *Effect of addition of large amount of activator on the formation of inhibitor-bound plasmin in the urokinase-activated plasma system*

Samples (200 μ l) of urokinase-activated plasma and clotted plasma with added 125 I-labelled Glu-plasminogen were prepared as those of Figs. 1 and 2 and incubated at 37°C. After 6 ks (plasma) or 3 ks (clotted plasma) 10 μ l of Tris buffer or 10 μ l of urokinase (1.4 μ M) was added to these samples, leading to no change or to a 25-fold increase in their amount of urokinase. Quantities denoted in the entries of the Table were determined immediately before (column A) and 3.6 ks after addition of Tris buffer (column B) or excess of urokinase (column C). This was done by quantification of radioactive bands in electrophoretic gels. Each value is the mean of three determinations. * indicates that 0.7 of the α_2 -plasmin-inhibitor-complexed plasmin was Glu-plasmin. In other instances only Lys-plasmin- α_2 -plasmin inhibitor complex was present. † denotes that the value is significantly different from other values either in plasma or in clotted plasma ($P < 0.01$).

	Plasma			Clotted plasma		
	A	B	C	A	B	C
Plasmin- α_2 -plasmin inhibitor complex, amount (pmol)	149*	154*	192†	178	178	201
Fraction of α_2 -plasmin inhibitor complexed to plasmin	0.62	0.64	0.80	0.74	0.74	0.84
Plasmin- α_2 -macroglobulin complex, amount (pmol)	53	62	168†	96	120	187†
Fraction of α_2 -macroglobulin complexed to plasmin	0.14	0.16	0.44	0.25	0.32	0.49
Fraction of the original Glu-plasminogen converted into complexed plasmin	0.42	0.45	0.75	0.57	0.62	0.81

with Figs. 1 and 2 here shows that a similar distribution of components was observed in the presence and the absence of Factor XIII. Autoradiography and quantification of components in the experiments of Figs. 1 and 2 were repeated with plasma and clotted plasma containing Factor XIII at 1.0 (=lysis time of the clotted plasma), 1.8 and 3.6 ks. Again, similar results were obtained in the presence and the absence of Factor XIII. In another experiment components were quantified in the same urokinase-activated clotted plasma at the time of clot lysis before and after removal of Factor XIII. No significant difference was found as regards the lysis time (approx. 1.2 ks) or the fractional distribution of plasminogen and plasmin-inhibitor complex forms when determined in triplicate. The transformation of plasminogen in the presence of Factor XIII was only studied after clot lysis, since the Factor XIII-catalysed cross-linking of fibrin made the urokinase-activated clotted plasma insoluble in acetic acid/urea in less than 0.6 ks.

Sequence of transformation of Glu-plasminogen effected by tissue-type plasminogen activator

The results obtained with pig tissue plasminogen activator and with melanoma-cell plasminogen activator were in all respects similar. Therefore only experiments with the latter activator are presented below.

Electrophoretic patterns. Autoradiographs of acetic acid/urea/polyacrylamide-slab-gel electrophoretograms showing the transformation of Glu-plasminogen in melanoma-cell plasminogen activator-activated plasma and clotted plasma are shown in Fig. 3. The radioactive zones migrated as those of Fig. 1. It was therefore assumed that they

were identical with those found in urokinase-activated systems.

Transformation of plasminogen in plasma. The reactions were studied at two different concentrations of activator (5.4 and 54 nM). The results were in all respects comparable with those obtained with urokinase (Figs. 1 and 2). At the low concentration (Fig. 4, □, ○, △ and ▽ symbols) plasminogen was converted into plasmin at a low rate, and fibrinogen was completely stable throughout the experiment. At the high concentration (Figs. 3 and 5) a considerable fraction of plasminogen was converted into plasmin and the concentration of clottable fibrinogen decreased rapidly.

Transformation of plasminogen in clotted plasma. This was studied only at the low activator concentration (Fig. 4, ■, ●, ▲ and ▼ symbols). The results were in most respects similar to those obtained with urokinase (Figs. 1 and 2). This was the case with regard to the pattern of Lys-plasminogen formation, the preferential binding of plasmin to α_2 -plasmin inhibitor and the progressive predominance of complexed Lys-plasmin before α_2 -plasmin inhibitor was saturated with plasmin (compare Figs. 3 and 4). The rate of plasmin formation was found to increase progressively until just before fibrin lysis. The increase was statistically significant ($P < 0.001$) with the pig tissue activator. After lysis of fibrin the rate of plasmin formation abruptly decreased and the amount of plasmin formed approached a value that was about half of the initial Glu-plasminogen concentration.

A fundamental difference was observed between the processes promoted by the tissue activator, at 5.4 nM, in plasma and in clotted

Fig. 3. Autoradiographs of gel slabs after acetic acid/urea/polyacrylamide-gel electrophoresis showing the transformation of Glu-plasminogen in plasma and clotted plasma activated by melanoma-cell plasminogen activator

Samples of plasma and clotted plasma with added ^{125}I -labelled Glu-plasminogen were activated by melanoma-cell plasminogen activator and processed as described in the legend to Fig. 1. The values above each gel track indicate the reaction time in ks. Letters a to f are defined in the legend of Fig. 1. Reaction mixtures contained initially plasminogen activator (plasma, 54 nM; clotted plasma, 5.4 nM; Glu-plasminogen 2.2 μM), clottable fibrinogen (6.4 μM), α_2 -plasmin inhibitor (1.3 μM) and α_2 -macroglobulin (1.9 μM). The plasma volume fraction was 0.87. Determinations were made in duplicate.

Table 2. Fibrin-binding of plasminogen and plasmin- α_2 -plasmin inhibitor complex in activator-activated clotted plasma. Samples of clotted plasma with added ^{125}I -labelled plasminogen were activated at 37°C by urokinase (2.3 nM) or by pig tissue activator (0.9 nM). At set times the amount of substance bound to fibrin and $A_{1\text{cm},280}$ of fibrin material (after solubilization in acetic acid/urea and dilution with NaOH) were determined as described in the Experimental section. The reaction mixture contained initially labelled + unlabelled Glu-plasminogen (2.5 μM), clottable fibrinogen (6.5 μM , $A_{1\text{cm},280} = 3.58$), α_2 -plasmin inhibitor (1.2 μM), α_2 -macroglobulin (1.9 μM). The plasma volume fraction was 0.85. The lysis times with both activators ranged between 1.71 and 1.95 ks. The values in parenthesis indicate the total amount of substance in the reaction mixtures at the specified times. In experiments indicated by asterisks (*) ^{125}I -labelled Lys-plasminogen was added to clotted plasma and labelled Glu-plasminogen was substituted with an equal amount of unlabelled Glu-plasminogen.

	Reaction time (ks)	n	Amount of substance bound to fibrin (mean $\pm$ S.E.M.) ($\mu\text{mol/l}$ of clot)			Amount of fibrin material (mean $\pm$ S.E.M.) ($A_{1\text{cm},280}$)
			Glu-plasminogen	Lys-plasminogen	Plasmin- α_2 -plasmin inhibitor complex	
Urokinase	0.12	3	0.11 $\pm$ 0.005 (2.4)	—	—	3.78 $\pm$ 0.02
	0.36	6	0.26 $\pm$ 0.021 (2.3)	—	0.022 $\pm$ 0.003 (0.18)	3.56 $\pm$ 0.08
	0.72	5	0.43 $\pm$ 0.028 (2.1)	—	0.036 $\pm$ 0.002 (0.42)	3.34 $\pm$ 0.11
	1.32	6	0.49 $\pm$ 0.024 (1.8)	0.15 $\pm$ 0.010 (0.16)	0.086 $\pm$ 0.011 (0.50)	2.96 $\pm$ 0.16
Tissue activator	0.12	4	0.059 $\pm$ 0.004 (2.4)	—	—	3.98 $\pm$ 0.12
	1.32	7	0.37 $\pm$ 0.019 (1.7)	0.14 $\pm$ 0.011 (0.19)	0.095 $\pm$ 0.007 (0.53)	3.42 $\pm$ 0.14
No activator		2	0.075 (2.5)	0.026 (0.13)	—	3.64
		2*		0.13 (0.50)	—	—
		2*			—	—

plasma. The rate of plasmin formation and the final amount of plasmin formed were much higher in the presence of fibrin. Fibrin lysed at 1.35 ks whereas fibrinogen was stable throughout the experiment. The rate enhancement of plasmin formation and plasmin proteolysis occurring in clotted plasma, as opposed to plasma, was far greater with melanoma-cell plasminogen activator than with urokinase.

Fibrin-binding of plasminogen and α_2 -plasmin inhibitor complex during transformation of Glu-plasminogen in clotted plasma

These aspects were investigated in clotted plasma activated by urokinase or by pig tissue plasminogen activator (Table 2). Initially, Glu-plasminogen was weakly bound to fibrin, but later in the course of the experiments its binding

Fig. 4. Concentration of plasminogen and plasmin-inhibitor complexes as a function of time in plasma and clotted plasma activated by melanoma-cell plasminogen activator at a concentration of 5.4 nM

Experimental conditions and composition of reaction mixtures are the same as indicated for Fig. 3. Concentrations of plasminogen and plasmin-inhibitor complexes in incubated samples of plasma and clotted plasma were determined as described in the legend to Fig. 2. Symbols are defined in the same legend. The decrease in Glu-plasminogen concentration as a function of time is not shown. As in Fig. 2, the sum of the concentrations of Glu-plasminogen, Lys-plasminogen and inhibitor-complexed plasmin was equal to the zero-time concentration of Glu-plasminogen. The concentration of clottable fibrinogen did not decrease within 7.2 ks.

increased significantly. Most of the Lys-plasminogen generated into clotted plasma was bound to fibrin. The fraction bound was 0.94 in the urokinase-activated system and 0.74 in the system with tissue activator (as calculated from the results of Table 2). Relatively less Lys-plasminogen bound to fibrin when added to clotted plasma without activator. The fraction bound was 0.20 and 0.26 when the amount of added Lys-plasminogen was respectively less (0.13 μM) and greater (0.50 μM) than that generated (0.16 and 0.19 μM) in activator-activated clotted plasma. The change in fibrin-binding of plasminogen during the activation process was associated with proteolysis of fibrin, as

Fig. 5. Concentration of plasminogen and plasmin-inhibitor complexes as a function of time in plasma activated by melanoma-cell plasminogen activator at a concentration of 54 nM

Experimental conditions and composition of reaction mixtures are those indicated for Fig. 3. Concentrations of plasminogen and plasmin-inhibitor complexes in incubated samples were determined as described in the legend of Fig. 2. Symbols are defined in the same legend. The decrease in Glu-plasminogen concentration as a function of time (not shown) was equal to the increase in concentration of inhibitor-complexed plasmin. The fibrinogen became unclottable between 1.2 and 7.2 ks. Determinations were made in duplicate.

can be seen from the decrease in A_{280} of acetic acid/urea-solubilized fibrin material with time ($P < 0.02$) (Table 2).

Only a small amount of plasmin- α_2 -plasmin inhibitor complex formed in activator-activated clotted plasma bound to fibrin (the fraction bound ranged between 0.09 and 0.18). The binding of plasmin- α_2 -macroglobulin complex was not determined, because the concentration of the complex before lysis of fibrin was too low to allow quantification. In agreement with the results obtained by Müllertz *et al.* (1984), no free plasmin could be detected when reduced samples of solubilized fibrin were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis followed by autoradiography.

Discussion

Transformation of plasminogen to plasmin may pass along two alternative pathways. One is an activator-catalysed conversion of Glu-plasminogen into Glu-plasmin followed by an autocatalytic conversion of Glu-plasmin into Lys-plasmin. The other is a plasmin-catalysed conversion of Glu-plasminogen into Lys-plasminogen followed by an

activator-catalysed conversion of Lys-plasminogen into Lys-plasmin. The pathway of plasminogen transformation depends on the environment (for review see Castellino, 1981).

Plasmin generated in plasma and clotted plasma became primarily complexed to α_2 -plasmin inhibitor and secondarily to α_2 -macroglobulin (Figs. 2, 4 and 5). This corroborated the results obtained by others (Müllertz, 1974; Collen, 1976; Müllertz & Clemmensen, 1976; Harpel, 1981) and was compatible with the high association rate constant for this inhibitor and plasmin (Christensen & Clemmensen, 1978; Wiman & Collen, 1978; Petersen & Clemmensen, 1981).

In plasma, plasminogen activation effected by both urokinase and tissue-type plasminogen activator followed the Glu-plasminogen-into-Glu-plasmin conversion pathway. This plasmin-catalysed Lys-plasminogen formation could not be demonstrated (Figs. 2, 4 and 5). About 70% of the generated plasmin was in its Glu- form as long as reactive α_2 -plasmin inhibitor was in molar excess of plasmin (Table 1 and Figs. 1 and 3) (Müllertz *et al.*, 1984). Fibrinogen has been shown to enhance the plasmin-catalysed conversion of Glu-plasminogen into its Lys- form (Lucas *et al.*, 1983). However, the present study indicated that fibrinogen does not decisively affect the pathway of plasminogen transformation in plasma.

In clotted plasma plasminogen activation initially followed the same Glu-plasminogen-into-Glu-plasmin conversion pathway as in plasma (Figs. 1 and 3). After a lag phase, however, a considerable amount of Lys-plasminogen and complexed Lys-plasmin was formed (Figs. 1-4), although reactive α_2 -plasmin inhibitor was in molar excess of plasmin. An increased rate of plasmin formation was not essential to Lys-plasminogen formation. Thus the rates of plasmin formation by the tissue-type plasminogen activator in plasma (Fig. 5) and clotted plasma (Fig. 4) were both considerable, and yet Lys-plasminogen was only detectable in the latter.

The decrease in rate of plasmin formation with time in plasma activated by both activator types was more pronounced than that observed with a reaction that followed first-order kinetics (Figs. 2, 4 and 5). This could be explained by progressive inactivation of activator by plasma inhibitors (Clemmensen & Christensen, 1976; Moroi & Aoki, 1976; Holmberg *et al.*, 1980; Korninger & Collen, 1981; Rijken *et al.*, 1983). Formation of fibrin had no immediate effect on the rate of urokinase-catalysed plasmin formation, though it produced the established enhancement of tissue-type-plasminogen-activator-catalysed plasmin formation (for references see the introduction). After a lag phase, when fibrin showed evident signs of

degradation and Lys-plasminogen was formed (Table 2), the rate of plasmin formation with urokinase increased in clotted plasma over that in plasma (Fig. 2) and the rate of plasmin formation with tissue-type plasminogen activator accelerated (Fig. 4). Altogether, these results and those obtained in purified systems (Suenson *et al.*, 1982, 1984) indicate that fibrin exerts its effector function by two different mechanisms. One mechanism starts to operate immediately after fibrin formation and only affects plasmin formation catalysed by tissue-type plasminogen activator. Probably fibrin primarily exerts its early effector function on the activator moiety of the enzyme-substrate complex, as tissue activator is selectively bound to fibrin (Thorsen *et al.*, 1972; Rijken & Collen, 1981) and as Glu-plasminogen initially is weakly bound to fibrin (Table 2) (Suenson *et al.*, 1984). The second enhancement mechanism is seen with both urokinase and tissue-type plasminogen activator and becomes operative when plasmin has cleaved a limited number of fibrin bonds (Table 2) (Suenson *et al.*, 1982, 1984). This leads to exposure of new site(s), which bind(s) Glu-plasminogen with higher affinity, probably inducing a change of conformation of Glu-plasminogen and a subsequent increase in Glu-plasmin and Lys-plasminogen formation. The latter conversion reaction irreversibly stabilizes plasminogen in a more reactive conformation, which has a higher fibrin-affinity (Table 2) (Thorsen, 1975) and is more readily activated to plasmin, both in the absence and in the presence of fibrin, than it is in its native conformation [Thorsen *et al.*, 1974; Castellino, 1981 (review); Hoylaerts *et al.*, 1982; Rånby, 1982]. Altogether, these events lead to enhanced plasmin formation. Fibrin and α_2 -plasmin inhibitor compete for the secondary-site binding of plasminogen and plasmin (Moroi & Aoki, 1977; Suenson & Thorsen, 1981). It is probable that the increase in affinity of plasminogen (and presumably plasmin) for fibrin on its early degradation (Table 2) leads to a decrease in rate of plasmin inactivation by α_2 -plasmin inhibitor and subsequently to an increase in the plasmin available for catalysing Lys-plasminogen formation and fibrin degradation.

It is well documented that complex-formation between lysine (or its analogues) and Glu-plasminogen changes the conformation of the latter so that the plasmin-susceptible bonds as well as the activator-susceptible bonds are cleaved at a higher rate than in the free pro-enzyme (for review see Castellino, 1981). Plasmin solely cleaves Lys-Xaa and Arg-Xaa peptide bonds in fibrin (for review see Henschen, 1983). It is possible that the appearance of C-terminal lysine residues contributes to the increase in fibrin-binding of Glu-plasminogen

and induces a change in conformation of Glu-plasminogen leading to increased rate of proteolytic cleavage of Glu-plasminogen by activator and plasmin.

The decrease in rate of Lys-plasminogen formation and the subsequent abrupt decrease in rate of plasmin formation observed after lysis of fibrin in clotted plasma (Figs. 2 and 4) is compatible with the interpretation that the effector function, emerging on initial plasmin-cleavage of fibrin, was compromised upon its further degradation (Wallén, 1977; Suenson *et al.*, 1982, 1984). An increase in rate of plasmin inactivation by α_2 -plasmin inhibitor may be an additional factor.

In conclusion, evidence is provided that plasmin-catalysed Lys-plasminogen formation is an important physiological feedback reaction during the process of fibrinolysis. It is substantiated that it is intimately linked to another positive feedback mechanism: the initial cleavage by plasmin of fibrin.

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