Identification of molecular forms of plasminogen and plasmin-inhibitor complexes in urokinase-activated human plasma

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Urokinase-activated human plasma was analysed by acetic acid/urea/polyacrylamide-gel electrophoresis. The bands representing plasminogen, the plasmin- α_2 -plasmin inhibitor and plasmin- α_2 -macroglobulin complexes were identified by immunoprecipitation with specific antibodies and by comparison with purified components. Plasminogen and the plasmin-inhibitor complexes were isolated from plasma or thrombin-clotted plasma containing ^{125}I -labelled Glu-plasminogen (residues 1–790) and urokinase. The plasma was kept at 37°C for 0.5 and 10 times the lysis time of the clotted plasma, the clotted plasma until lysis. The plasmin heavy chain from the plasmin-inhibitor complexes was subsequently prepared. Only in one case could a low-grade proteolytic conversion of Glu- forms into Lys/Met/Val- forms (residues 77–790, 68–790 and 78–790 respectively) during the preparations be detected. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and *N*-terminal sequence analysis of the purified plasminogen and plasmin heavy chain showed the following. The plasminogen in plasma was on the Glu- form. Glu-plasmin constituted 0.74 and 0.58 of the plasmin bound to the α_2 -plasmin inhibitor in plasma after brief and prolonged activation respectively. The rest was Lys/Met/Val-plasmin. The clotted plasma contained about equal amounts of Glu-plasminogen and Lys/Met/Val-plasminogen, and predominantly Lys/Met/Val-plasmin complexed to α_2 -plasmin inhibitor and α_2 -macroglobulin. The results of the analysis of the purified material substantiated the identity of radioactive protein bands in the gel after acetic acid/urea/polyacrylamide-gel electrophoresis.

The resolution of fibrin in the organism involves the conversion of plasminogen into plasmin (EC 3.4.21.7) catalysed by plasminogen activator and the subsequent degradation of fibrin catalysed by plasmin. Plasmin generated in plasma primarily forms a complex with α_2 -plasmin inhibitor (α_2 -antiplasmin), and secondarily it forms a complex with α_2 -macroglobulin (Müllertz, 1974; Müllertz & Clemmensen, 1976; Collen, 1976).

Abbreviations used: KU unit, kallikrein-inhibitor unit; NIH unit, National Institutes of Health unit; aprotinin, bovine pancreatic trypsin inhibitor (Kunitz inhibitor); 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); SDS, sodium dodecyl sulphate.

The plasminogen activator catalyses the cleavage of the Arg₍₅₆₀₎-Val₍₅₆₁₎ bond in Glu-plasminogen, forming plasmin (Robbins *et al.*, 1967; Wiman, 1977; Sottrup-Jensen *et al.*, 1978a). This cleavage divides the molecule in the *N*-terminal heavy (A-) and *C*-terminal light (B-) chain linked by two disulphide bonds. Before or after cleavage of the Arg₍₅₆₀₎-Val₍₅₆₁₎ bond, plasmin may catalyse the cleavage of the Lys₍₇₆₎-Lys₍₇₇₎ (Arg₍₆₇₎-Met₍₆₈₎, Lys₍₇₇₎-Val₍₇₈₎) bond in Glu-plasminogen or in the analogous part of Glu-plasmin, forming Lys/Met/Val-plasminogen or Lys/Met/Val-plasmin (Claeys *et al.*, 1973; Violand & Castellino, 1976). This cleavage leads to the release of the *N*-terminal residues.

The object of the present work is to identify molecular forms of plasminogen and molecular forms of plasmin complexed to α_2 -plasmin inhibitor and α_2 -macroglobulin in plasma, in parti-

cular the identity of compounds with different mobilities in acetic acid/urea/polyacrylamide-gel electrophoresis, in order to use this analytical tool for a quantitative determination of the compounds as a function of time in a subsequent study (Thorsen *et al.*, 1984).

Experimental

Materials

Buffers and chemicals. Tris buffer (10.15), pH 7.70 (20°C), and gelatin Tris buffer (10.15), pH 7.70 (20°C), have been described previously (Thorsen, 1975).

Dithioerythritol, *p*-nitrophenyl *p*'-guanidinobenzoate and sodium iodoacetate were from Sigma Chemical Co., St. Louis, MO, U.S.A. *p*-Nitrophenyl *p*'-guanidinobenzoate was dissolved as described by Kézdy & Kaiser (1970). Ammonium heparinate (146 i.u./mg) was from Leo Pharmaceuticals, Ballerup, Denmark.

Proteins. Aprotinin (Trasylol) (courtesy of Dr. Philipp, Bayer, Leverkusen, Germany), bovine thrombin (96 NIH units/mg) and human urokinase (EC 3.4.21.31, 7700 Ploug units/mg) have been described previously (Thorsen, 1975). SDS/polyacrylamide-gel electrophoresis followed by zymographic analysis (Granelli-Piperno & Reich, 1978) demonstrated the two forms of urokinase with M_r 31 000 and 54 000. Glu-plasminogen, aprotinin-inhibited Glu-plasmin, Lys-plasminogen, aprotinin-inhibited Lys-plasmin and the plasminogen-binding form of α_2 -plasmin inhibitor were prepared as described previously (Clemmensen *et al.*, 1981; Suenson & Thorsen, 1981; Thorsen *et al.*, 1981).

Human plasma. This was prepared from citrated out-dated human donor blood. Plasma was centrifuged at 23 000 *g* at 4°C for 2 h, and the clear plasma was siphoned off.

Antibodies against human proteins. Specific rabbit immunoglobulins against plasma inhibitors, plasminogen and urokinase were those previously reported (Müllertz, 1974; Clemmensen & Christensen, 1976; Clemmensen *et al.*, 1981).

Methods

Determination of the concentration of protein. The concentration of purified plasminogen forms was calculated from the absorbance at 280 nm (Thorsen & Müllertz, 1974) of purified α_2 -plasmin inhibitor by active-site titration (Müllertz & Clemmensen, 1976) of Glu-plasminogen or inhibitors in plasma by electroimmunoassay (Laurell, 1972), with purified Glu-plasminogen, purified α_2 -plasmin inhibitor or α_2 -macroglobulin in Protein-Standard-Plasma (Behringwerke, Marburg, Germany) being used for calibration.

Labelling of plasminogen with ^{125}I . This was performed as previously described (Thorsen *et al.*, 1981). The specific radioactivity ranged between 1 and 11 mCi/ μmol of plasminogen. The incorporation of iodine into plasminogen was less than 1 g-atom/mol.

Polyacrylamide-gel electrophoresis in the presence of SDS and of acetic acid/urea. These were performed as described previously (Suenson & Thorsen, 1981), except that gels for electrophoresis in the presence of acetic acid/urea contained an upper layer (length around 10 mm) with acrylamide (48.7 g/l) and *NN'*-methylenebisacrylamide (1.3 g/l). This allowed high-molecular-mass proteins to penetrate into the gel.

Autoradiography and quantification of ^{125}I -labelled plasminogen derivatives by polyacrylamide-gel electrophoresis. Both were performed as described previously (Suenson & Thorsen, 1981). Plasma was either applied directly with purified material run simultaneously to guide the excision of gel bands, or washed precipitate was applied, prepared by immunoprecipitation by an excess of anti-plasminogen immunoglobulin. The recovery of radioactivity was 0.96.

The precision of the method was estimated both with plasma and with preparations. The results were as follows (means $\pm$ s.d.): plasmin- α_2 -plasmin inhibitor, in plasma, anodic, cathodic and total, 0.21 ± 0.01 , 0.12 ± 0.02 and 0.33 ± 0.02 respectively, and in preparations 0.54 ± 0.06 , 0.30 ± 0.06 and 0.84 ± 0.05 respectively; plasminogen, in plasma, anodic, cathodic and total, 0.37 ± 0.03 , 0.03 ± 0.01 and 0.33 ± 0.02 respectively, and in preparations 0.88 ± 0.03 , 0.04 ± 0.01 and 0.92 ± 0.03 respectively. The number of duplicates (*n*) was 11 for plasma and 12 for preparations. Means of triplicates were used. The s.d. of the ratios in Table 1 was calculated from the coefficients of variation (*V*) ($V_{y/x}^2 = V_y^2 + V_x^2$), and differences were evaluated by Student's *t* test.

The conversion of labelled Glu-plasminogen into Lys-plasminogen did not result in any loss of radioactivity (*n* = 4, *P* > 0.1; Student's *t* test), when estimated as described above by acetic acid/urea/polyacrylamide-gel electrophoresis of samples containing Glu-plasminogen and of Lys-plasminogen prepared from Glu-plasminogen by treatment with trace amounts of urokinase (Thorsen & Müllertz, 1974). The radioactivity of the light chain as a fraction of the radioactivity of the heavy chain + light chain of aprotinin-inhibited Glu-plasmin [mean $\pm$ s.d. (*n*)] was 0.41 ± 0.015 (6).

The total radioactivity in the gel track was more than 94% of that of the sample applied.

Preparation of ^{125}I -labelled plasmin- α_2 -plasmin inhibitor complex from purified components. Labelled plasminogen was activated by urokinase at a molar

excess of α_2 -plasmin inhibitor (Wiman & Collen, 1979). The mixture in Tris buffer contained plasminogen (7 μ M), α_2 -plasmin inhibitor (11–13 μ M), and urokinase (8×10^5 Ploug units/l). It was incubated for 30 min (Lys-plasminogen) or 60 min (Glu-plasminogen) at 37°C, and the reaction was quenched by addition of 2 vol. of acetic acid (1.75 M)/urea (9 M). It was stored at 4°C and used within 2 h. Plasmin heavy chains from complexes prepared from Glu-plasminogen and Lys-plasminogen had M_r 65 000 and 60 000 respectively, indicating that the *N*-terminal peptide fragments were not released from Glu-plasminogen at an excess of α_2 -plasmin inhibitor (Wiman & Collen, 1979).

Identification by immunoprecipitation of α_2 -plasmin inhibitor and α_2 -macroglobulin complexed to 125 I-labelled plasmin. Plasma without Factor XIII with added 125 I-labelled Glu-plasminogen was mixed with urokinase in gelatin/Tris buffer, or with a mixture of urokinase and thrombin as described (Thorsen *et al.*, 1984), and incubated at 37°C. After a given time (after lysis of fibrin in clotted plasma) aprotinin was added to 17 μ M, ammonium heparinate (plasma only) to 36 units/l, and specific rabbit immunoglobulins against α_2 -plasmin inhibitor (3 vol.) or against α_2 -macroglobulin (1 vol.). After incubation for 30 min at 37°C and 18 h at 4°C, the immunoprecipitates were collected by centrifugation, washed in NaCl (150 mM) at 4°C and resuspended in acetic acid (1.25 M)/urea (6 M). The plasma samples (before and after immunoprecipitation) diluted in 2 vol. of acetic acid (1.75 M)/urea (9 M), and the solubilized immunoprecipitates were subjected to acetic acid/urea/polyacrylamide-gel electrophoresis followed by autoradiography. The supernatants after immunoprecipitation did not contain inhibitor-antigenic material when tested by electroimmunoassay.

Isolation of plasminogen and plasmin-inhibitor complexes from urokinase-activated plasma and clotted plasma. To plasma was added 125 I-labelled Glu-plasminogen and urokinase. One specimen was coagulated by thrombin (10^3 NIH units/l) (CP) and kept at 37°C until lysis (about 1.4 ks). Two specimens were kept as plasma at 37°C, one for 0.5 lysis times (P1) and the other for 10 lysis times (P2) of that of CP. The final concentration of urokinase was 25×10^3 – 40×10^3 Ploug units/l, adjusted to yield a lysis time about 1.4 ks of thrombin-coagulated aliquots. The ratio of radiolabelled plasminogen to total plasminogen was 0.03 (P1 and CP) and 0.001 (P2). The volumes of plasma (undiluted) processed were 833 ml (P1), 1618 ml (P2), and 810 ml (CP). The activation was stopped by addition of specific rabbit immunoglobulin against urokinase, in amounts found to prevent the lysis of clotted samples of plasma (1 ml per 5000 Ploug

units). Then the material was diluted with 2 vol. of ice-cold sodium phosphate buffer (100 mM), pH 7.4, containing aprotinin (5 μ M) and *p*-nitrophenyl *p*'-guanidinobenzoate (10 μ M). The diluted material (P1 and CP) was divided into nine 350 ml portions, quickly frozen in a bath of solid- CO_2 /acetone, and stored at -20°C . Three portions were processed simultaneously at 4°C. Each portion was applied to a column (Pharmacia K 26/40) containing 60 ml of lysine-Sepharose 4B equilibrated with sodium phosphate buffer (100 mM), pH 7.4, containing aprotinin (5 μ M) and *p*-nitrophenyl *p*'-guanidinobenzoate (10 μ M). The gel was washed with the equilibration buffer until $A_{1\text{cm},280}$ was less than 0.02. The 125 I-labelled material was eluted with the same buffer, but containing 6-aminohexanoic acid (20 mM). The eluted fractions were collected in 0.2 vol. of acetic acid (10 M) to give a final pH of 3.4. The radioactive fractions from the three columns were pooled and concentrated to a volume of about 30 ml and subjected to gel filtration on a column (Pharmacia K 50/100) containing 1700 ml of Ultrogel AcA-34 (LKB, Stockholm, Sweden) and equilibrated and eluted with glycine (50 mM)/HCl (50 mM)/NaCl (300 mM), pH 3.4, or with acetic acid (2.5 M)/sodium acetate (100 mM)/NaCl (200 mM), pH 3.4. The radioactivity and the protein (measured by A_{280}) were eluted in a minor and two major peaks that contained the plasmin- α_2 -macroglobulin complex, the plasmin- α_2 -plasmin inhibitor complex and the plasminogen respectively. Fractions of the central parts of the peaks were pooled and concentrated by ultrafiltration to $A_{1\text{cm},280} = \sim 1$ (plasmin-inhibitor complexes) or to $A_{1\text{cm},280} = 2.5$ (plasminogen). The buffer of the plasminogen pool was exchanged with acetic acid (0.5 M) by Sephadex G-25 gel filtration, concentrated and freeze-dried. The pools of the plasmin-inhibitor complexes were stored at -20°C .

The lysine-Sepharose step of P2 was done as follows. Plasma (2 litres) was stirred with lysine-Sepharose (600 ml) for 1 h at 4°C and washed on a Buchner funnel with phosphate buffer (100 mM), pH 7.4. The gel was packed in a column (Pharmacia K 50/60) and eluted with the same buffer (1800 ml) containing a linear gradient of 6-aminohexanoic acid from 0 to 20 mM.

Determination of the amount of substance of plasminogen derivatives in the plasma samples and in the purified materials of the preparative experiments. The concentration of plasminogen in the purified plasminogen preparation and the radioactivity determined and the molar radioactivity were calculated. The total amount of substance of plasminogen derivatives in any material was calculated by dividing the total radioactivity of the material by the molar radioactivity of plasminogen. The

amount of substance of each component was then calculated from the distribution of the radioactivity in the gel after electrophoresis as described above. This was possible because the molar radioactivity of the various forms of plasminogen and plasmin did not differ.

Preparation of the plasmin heavy chain from the plasmin-inhibitor complexes. This was done as described by Wiman & Collen (1979), after adjustment of the pH of the acid pools with Tris base to 8.4. The solutions were desalted by Sephadex G-25 gel filtration in acetic acid (0.5M), concentrated by ultrafiltration and freeze-dried.

Amino acid sequence determination. Samples (2–10nmol) were subjected to manual Edman degradation similar to the procedure described by Skorstensgård *et al.* (1982), or to automated Edman degradation in a Beckman 890 C instrument as in Sottrup-Jensen *et al.* (1981). Phenylthiohydantoin derivatives of amino acids were analysed by high-performance liquid chromatography on columns of Spherisorb 5 S (octadecylsilane-coated) as described by Sottrup-Jensen *et al.* (1980).

Results and discussion

Electrophoretic mobility of plasminogen derivatives and identification of plasmin-inhibitor complexes in plasma

Acetic acid/urea/polyacrylamide-gel electrophoresis of urokinase-activated plasma and clotted plasma with ^{125}I -labelled Glu-plasminogen displayed four radioactive zones in the gel. From the cathode towards the anode they were as follows: zones 1 and 2 represented plasminogen, zone 3 represented the plasmin- α_2 -plasmin inhibitor complex and zone 4, close to the site of application, represented the plasmin- α_2 -macroglobulin complex (Fig. 1, track *a*). The evidence is as follows.

Zone 1 was a double band migrating as Lys-plasminogen (cathodic plasminogen) (Fig. 1, tracks *a* and *c*). Zone 2 was a double band migrating as Glu-plasminogen (anodic plasminogen) (Fig. 1, tracks *a* and *c*). The double bands represented the two carbohydrate variants of the proenzyme (Walther *et al.*, 1975).

Zone 3 displayed three bands. Two of the bands

Fig. 1. Autoradiographs of gels and gel stained for protein after acetic acid/urea/polyacrylamide-gel electrophoresis showing the mobility of free and inhibitor-bound ^{125}I -labelled plasminogen derivatives in plasma and after purification

The Figure shows the patterns obtained after electrophoresis of the following materials: clotted plasma activated by urokinase (10^5 Ploug units/l, lysis time = 0.30 ks) at 37°C for 1.20 ks (*a*) or 0.30 ks (*b*); mixture of unlabelled Glu-plasminogen (anodic) and Lys-plasminogen (cathodic) (*c*); clotted plasma activated by urokinase (3×10^4 Ploug units/l, lysis time = 1.26 ks) for 1.20 ks (*d*); plasma activated by urokinase (2×10^4 Ploug units/l) for 1.20 ks (*e*) or 12.0 ks (*f*); plasmin- α_2 -plasmin inhibitor complex prepared by activation of ^{125}I -labelled Glu-plasminogen (*g*) or Lys-plasminogen (*h*) by urokinase in the presence of excess of inhibitor. Abbreviations: Pg, plasminogen; P- α_2 PI, plasmin- α_2 -plasmin inhibitor complex; P- α_2 M, plasmin- α_2 -macroglobulin complex. The same gel slab was used in runs (*a*), (*b*) and (*c*) and in runs (*d*), (*e*), (*f*), (*g*) and (*h*). Track (*c*): gel slab stained for protein. Other tracks: autoradiographs.

were observed in plasma and in activated clotted plasma at the time of clot lysis (Fig. 1, tracks *b*, *d*, *e* and *f*). They had the same relative mobilities (anodic and cathodic) as purified complexes of the α_2 -plasmin inhibitor assumed to contain Glu-plasmin and Lys-plasmin respectively (Fig. 1, tracks *g* and *h*). Activated clotted plasma incubated 6 times its lysis time displayed the cathodic band described above (Fig. 1, track *a*) and in addition another band that migrated somewhat further towards the cathode. The latter band increased with the time after clot lysis (Thorsen *et al.*, 1984). All bands of zone 3 were removed by immunoprecipitation with specific rabbit immunoglobulin against α_2 -plasmin inhibitor and were recovered in the immunoprecipitate (Fig. 2). Zone 4 was removed by immunoprecipitation with specific rabbit immunoglobulin against α_2 -macroglobulin and was recovered in the immunoprecipitate (Fig. 2).

The mobility and the pattern of the bands of zone 3 depended on the length of preincubation of the sample in acetic acid/urea. After a short preincubation (<10 min at 5°C) the bands migrated as shown in Figs. 1 and 2. After a long preincubation (>24 h at 5°C) the total zone had a more-

anodic mobility, and only two bands, an anodic and a cathodic band, were seen, the former corresponding to the anodic band, the latter to the cathodic bands, observed after a short preincubation (Fig. 3). The 'short incubation pattern' was often difficult to reproduce with purified material.

Purification of the plasminogen and the plasmin-inhibitor complexes. These experiments were done without removal of Factor XIII as opposed to those of the following paper (Thorsen *et al.*, 1984). Plasma with added ^{125}I -labelled Glu-plasminogen was activated with urokinase for a short period (P1) and a long period (P2) and until complete lysis of clotted plasma (CP). The distribution of the plasminogen and plasmin-inhibitor complexes after acetic acid/urea/polyacrylamide-gel electrophoresis was similar to that demonstrated in Fig. 1 (tracks *e*, *f* and *b*). The ^{125}I -labelled plasminogen and the ^{125}I -labelled plasmin-inhibitor complexes were purified from P1, P2 and CP. The pattern of the gels after acetic acid/urea/polyacrylamide-gel electrophoresis of these preparations is shown in Fig. 4. Whereas plasminogen was very pure, the preparations of the plasmin-inhibitor complexes contained some protein impurities. Further purifi-

Fig. 2. Autoradiographs of gels after acetic acid/urea/polyacrylamide-gel electrophoresis identifying ^{125}I -labelled plasmin-inhibitor complexes in plasma by immunoprecipitation

Plasma or clotted plasma was activated by urokinase (2×10^4 Ploug units/l) at 37°C. After a given time the plasmin- α_2 -plasmin inhibitor complex or the plasmin- α_1 -macroglobulin complex was immunoprecipitated by specific rabbit immunoglobulin against the inhibitor. (A) Plasma incubated for 3.00 ks, immunoprecipitation of plasmin- α_2 -plasmin inhibitor complex (anodic + cathodic); (B) clotted plasma incubated for 6.00 ks, immunoprecipitation of plasmin- α_1 -macroglobulin complex. (a) Plasma before immunoprecipitation; (b) immunoprecipitate; (c) plasma after immunoprecipitation. The lysis time was 1.11 ks in (B). Abbreviations: see the legend to Fig. 1.

Fig. 3. Protein pattern of gel slabs after acetic acid/urea/polyacrylamide-gel electrophoresis of the plasmin- α_2 -plasmin inhibitor complex after a short and a long preincubation in acetic acid/urea

The complex was prepared from plasma incubated for 0.5 lysis times (P1) and from clotted and lysed plasma (CP) (see the Experimental section). A sample was incubated at 5°C for 5 min (A) and 24 h (B) in acetic acid/urea before electrophoresis. The gel slabs were stained for protein. Abbreviations: see the legend to Fig. 1.

cation was abstained from, because it increased the risk of proteolytic degradation. Furthermore, the impurities were removed by the subsequent isolation of the plasmin heavy chain. The radioactive fraction of the main component was 0.92–0.97 (plasminogen, plasmin- α_2 -plasmin inhibitor complex) and 0.83 (plasmin- α_2 -macroglobulin complex from CP).

Table 1 shows the fractional distribution and the total amount of substance of the components both in plasma and in the partially purified preparations.

The recoveries were low, in particular those of the plasmin-inhibitor complexes from the plasma experiments (P1 and P2). The major losses occurred in the gel-filtration steps. The (cathodic form)/(anodic + cathodic forms) ratios of the plasmin- α_2 -plasmin inhibitor and of plasminogen

Table 1. Distribution of different forms of plasminogen and of plasmin-inhibitor complexes and the total amount of substance in three urokinase-activated plasma samples and in partially purified preparations

Component	P1			P2			CP		
	Sample	Material	...	Plasma	P- α_2 PI prepn.	Pg prepn.	Plasma	P- α_2 PI prepn.	Pg prepn.
A Plasmin- α_2 -macroglobulin		0.03		0.01	0	0.06	0.02	0.01	0.83
B Plasmin- α_2 -plasmin inhibitor, anodic		0.14		0.71	0.01	0.25	0.09	0	0.05
C Plasmin- α_2 -plasmin inhibitor, cathodic		0.05		0.23	0.02	0.18	0.30	0.02	0.07
D Plasminogen, anodic		0.78		0.04	0.93	0.45	0.29	0.51	0.02
E Plasminogen, cathodic		0.01		0.01	0.04	0.06	0.21	0.46	0.03
A + B + C + D + E		1615		18	497	4150	1633	300	29
C/(B + C)		0.26		0.24		0.42	0.77	0.83	
E/(D + E)		0.01		0.04*		0.12	0.42	0.47	
				Fractional distribution		Amount of substance (nmol)		Molar ratio	
				0.02		182		0.42	
				0.54		747		0.08	
				0.39					
				0.04					
				0.01

Fig. 4. Acetic acid/urea/polyacrylamide-gel electrophoresis showing the mobility of free and inhibitor-bound plasminogen derivatives in seven purified preparations

Plasma or clotted plasma was activated by urokinase followed by purification of components by affinity chromatography on lysine-Sepharose 4B and gel filtration on Ultrogel AcA-34 as described in the Experimental Section. Plasmin- α_2 -plasmin inhibitor complex ($P-\alpha_2PI$), plasminogen (Pg) and plasmin- α_2 -macroglobulin complex ($P-\alpha_2M$) were from plasma incubated 0.5 lysis times ($P1$) and 10 lysis times ($P2$) and clotted plasma incubated until complete lysis (CP). The samples were diluted with 2 vol. of acetic acid (1.75M)/urea (9M) and incubated for 24h at room temperature. (a)–(c) Preparation of $P-\alpha_2PI$ from $P1$ (a), $P2$ (b) and CP (c); (d)–(f) preparation of Pg from $P1$ (d), $P2$ (e) and CP (f); (g) preparation of $P-\alpha_2M$ from CP (separate experiment).

(Table 1) were of interest, because an increase during the preparative procedure would indicate proteolytic (by plasmin) degradation. In one case ($P1$) there was a significant but small increase in the amount of degraded plasminogen. The other increases (if any) were insignificant.

Identification of the molecular forms of plasminogen

Plasminogen purified from $P1$ and $P2$, which predominantly contained the anodic form (Table 1), mainly migrated as Glu-plasminogen in acetic acid/urea/polyacrylamide-gel and SDS/polyacrylamide-gel electrophoresis (Figs. 4 and 5) and displayed the dominating *N*-terminal sequence Glu-Pro (Table 2). Plasminogen purified from CP , which contained about equal amounts of anodic and cathodic forms (Table 1), migrated as Glu- and Lys-plasminogen in acetic acid/urea/polyacrylamide and SDS/polyacrylamide-gel electrophoresis (Figs. 4 and 5) and displayed the *N*-

terminal sequence Glu-Pro (fraction = 0.4), and the sequences Met/Lys-Val/Val-Tyr (fraction = 0.6) (Table 2). These sequences are characteristic for Glu-plasminogen and Lys/Met/Val-plasminogen respectively (Sottrup-Jensen *et al.*, 1978a,b).

Hence anodic plasminogen in plasma and clotted plasma and cathodic plasminogen demonstrated in clotted plasma by acetic acid/urea/polyacrylamide-gel electrophoresis (Fig. 1) are Glu- and Lys/Met/Val-plasminogen respectively.

Free Glu-plasmin and Lys/Met/Val-plasmin migrate as their analogous plasminogen forms in acid/urea/polyacrylamide-gel electrophoresis of non-reduced samples (Suenson & Thorsen, 1981). The amount of free plasmin in the urokinase-activated plasma and clotted plasma was small, as plasmin light chain was present in only trace amounts in reduced samples of the purified plasminogen. This is compatible with the fact that α_2 -

Fig. 5. SDS/polyacrylamide-gel electrophoresis showing the mobility of plasminogen isolated from the plasma systems

Plasminogen (reduced samples): Glu-plasminogen (a); Lys-plasminogen (b); plasminogen from CP (c), from P1 (d) and from P2 (e). For definitions of CP, P1 and P2 see the legend to Table 1.

plasmin inhibitor binds plasmin rapidly and strongly (Christensen & Clemmensen, 1978; Wiman & Collen, 1978).

Identification of molecular forms of plasmin heavy chain in the plasmin- α_2 -plasmin inhibitor complex

The fractions of heavy chain recovered from the complex in the preparations were 0.53 (P1), 0.43 (P2) and 0.43 (CP). The plasmin heavy chain isolated from the complex of P1, which predominantly contained the anodic form of the complex (Table 1), displayed the dominating *N*-terminal sequence characteristic for Glu-plasmin (Table 2) (Sottrup-Jensen *et al.*, 1978*a,b*). The plasmin heavy chain isolated from the complex of CP, which predominantly contained the cathodic forms of the complex (Table 1), displayed predominantly the *N*-terminal sequence characteristic for Lys/Met/Val-plasmin (Table 2) (Sottrup-Jensen *et al.*, 1978*a,b*). Only an incomplete separation of the Glu- and the Lys/Met/Val-plasmin heavy chain by SDS/polyacrylamide-gel electrophoresis was achieved. However, the plasmin heavy chain of the complex prepared from P1 and CP migrated mainly as the Glu form and the Lys/Met/Val-form respectively (results not shown). After prolonged activation of plasma (P2), somewhat more of the anodic (0.58) than the cathodic form (0.42) of the complex was formed (Table 1). About equal amounts of the *N*-terminal sequences (Table 2) were observed. The band 2 of the cathodic plasmin- α_2 -plasmin inhibitor complex (Fig. 1) appeared only when most of the α_2 -plasmin inhibitor was complexed to plasmin (Thorsen *et al.*, 1984). It was not present in

Table 2. Expected and determined *N*-terminal amino acid sequences of plasmin from the plasmin-inhibitor complexes and plasminogen

The presumed distribution of the *N*-terminal sequences was calculated from the distribution of the components by acetic acid/urea/polyacrylamide-gel electrophoresis (Table 1) on the presupposition that cathodic plasminogen, and plasmin from plasmin- α_2 -macroglobulin and from cathodic plasmin- α_2 -plasmin inhibitor complex, have *N*-terminal Met/Val/Lys, whereas anodic plasminogen, and plasmin from anodic plasmin- α_2 -plasmin inhibitor, have *N*-terminal Glu. The fractional distribution was calculated from *N*-terminal sequence analysis of the plasmin heavy chain isolated from the plasmin-inhibitor complexes and from plasminogen. For abbreviations see the legend to Table 1.

Preparation	Source	Fractional distribution							
		Expected				Determined			
		Glu-Pro	Met-Arg	Val-Tyr	Lys-Val	Glu-Pro	Met-Arg	Val-Tyr	Lys-Val
Plasmin- α_2 -macroglobulin	CP	0.07		0.93		0.2		0.8	
Plasmin- α_2 -plasmin inhibitor	P1	0.75		0.25		0.8		0.2	
	P2	0.58		0.42		0.5		0.5	
	CP	0.18		0.82		0.1		0.9	
Plasminogen	P1	0.94		0.06		> 0.9		0	
	P2	0.88		0.13		> 0.9		0	
	CP	0.51		0.49		0.4		0.6	

the purified plasmin-inhibitor complex assumed to contain Lys-plasmin (see the Experimental section) (Fig. 1). The band probably reflected a plasmin-catalysed cleavage of peptide bonds in the plasmin light chain or in the α_2 -plasmin inhibitor of the complex (Wiman & Collen, 1979).

Plasma contains a plasminogen-binding and a non-plasminogen-binding form of the α_2 -plasmin inhibitor (Clemmensen *et al.*, 1981). These forms have identical mobilities in acid/urea/polyacrylamide-gel electrophoresis when they are complexed to Glu- or Lys-plasmin.

Hence the anodic and the cathodic forms of plasmin- α_2 -plasmin inhibitor complex demonstrated in plasma and clotted plasma by acid/urea/polyacrylamide-gel electrophoresis (Fig. 1) contain Glu-plasmin and Lys/Met/Val-plasmin respectively.

Identification of plasmin heavy chain in the plasmin- α_2 -macroglobulin complex

The complex was recovered from CP only in amounts sufficient for isolation of the plasmin heavy chain. The molar fraction of heavy chain recovered from the purified complex from CP was 0.34. The heavy chain migrated predominantly as Lys-plasmin heavy chain in SDS/polyacrylamide-gel electrophoresis and displayed the dominating N-terminal sequence, Lys-Val, characteristic for Lys-plasmin (Table 2). The sequence Glu-Pro was also observed, in a relative amount of about 0.2 (Table 2).

Hence the plasmin- α_2 -macroglobulin complex (CP) mainly contained Lys/Met/Val-plasmin.

Concluding remarks

Different molecular forms of plasminogen and different molecular forms of plasmin complexed to α_1 -plasmin inhibitor and α_2 -macroglobulin were identified in urokinase-activated plasma and clotted plasma. Plasminogen derivative bands with the same mobilities in acid/urea/polyacrylamide-gel electrophoresis appeared when plasma or clotted plasma was activated by high-fibrin-affinity-plasminogen activator (human melanoma cell activator, pig tissue activator) or by the low-fibrin-affinity-plasminogen activator urokinase (Thorsen *et al.*, 1984). It was therefore assumed that they had the same identities.

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