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Identification and Some Properties of a New Fast-Reacting Plasmin Inhibitor in Human Plasma

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Fresh plasma was seeded with trace amounts of highly purified biologically intact iodine-labelled plasminogen and the plasmin · inhibitor complexes formed after activation with streptokinase or urokinase separated by gel filtration. Two radioactive peaks were observed, the first one eluted in the void volume and the second one just before the 7-S globulin peak. In incompletely activated samples, the second peak was always predominant over the first one.

Both components were purified with high yield by a combination of affinity chromatography on lysine-agarose and gel filtration, and investigated by dodecylsulphate-polyacrylamide gel electrophoresis and immunoelectrophoresis. Neither component reacted with antisera against α_1-antitrypsin, antithrombin III, C_1-esterase inhibitor, inter-α-trypsin inhibitor or α_1-antichymotrypsin. The component of the first peak appeared to be a complex between plasmin and α_2-macroglobulin which reacted with antisera against human plasminogen and against α_2-macroglobulin.

The component of the second peak had a molecular weight (M_r) of 120000–140000 by dodecylsulphate-polyacrylamide gel electrophoresis and upon reduction displayed a doublet band with an M_r of 65000–70000 and a band with M_r 11000. It reacted with antisera against plasminogen and with antisera raised against this complex and absorbed with purified plasminogen. The latter antisera reacted with a single component in plasma which is different from the above-mentioned plasma protease inhibitors. Specific removal of this component from plasma by immuno-absorption resulted in disappearance of the fast-reacting antiplasmin activity whereas α_2-macroglobulin was found to represent the slower-reacting plasmin-neutralizing activity. In the presence of normal plasma levels of these proteins, the specific removal or absence of α_1-antitrypsin, antithrombin III or C_1-esterase inhibitor did not alter the inactivation rate of plasmin when added to plasma in equimolar amounts to that of plasminogen.

It is concluded that only two plasma proteins are important in the binding of plasmin generated by activation of the plasma plasminogen, namely a fast-reacting inhibitor which is different from the known plasma protease inhibitors and which we have provisionally named antiplasmin, and α_2-macroglobulin, which reacts more slowly.

There are at least five well-defined plasma proteins which inhibit plasmin in a purified system, namely α_2-macroglobulin, α_1-antitrypsin, inter-α-trypsin inhibitor, antithrombin-III · heparin complex and C_1-esterase inhibitor [1].

The role of these inhibitors in the inactivation of plasmin, formed in its biological environment plasma, is not well understood. It has long been accepted that there are essentially two functionally important plasmin inhibitors in plasma, an immediately reacting one and a slow-reacting one [2], identical with α_2-macroglobulin and α_1-antitrypsin [3,4], respectively.

Nilehn and Ganrot [5] studied human sera obtained before and during streptokinase therapy with the use of gel filtration and immunoelectrophoresis with specific antibodies. These authors found that the plasminogen antigen shifted from its normal symmetrical elution just before the albumin peak to an asymmetrical elution pattern with a peak at the void volume and a tailing slope. They concluded that the plasmin formed was bound to α_2-macroglobulin and not to α_1-antitrypsin and ascribed the tailing of plasminogen antigen during gel filtration to dissociation of the plasmin · α_2-macroglobulin complex.

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Note. Enzyme units have been defined by the Committee on Thrombolytic Agents (CTA).

Enzymes. Plasmin (EC 3.4.21.7); urokinase (EC 3.4.99.26); streptokinase (EC 3.4.99.22).

Müllertz studied the activation products of human plasminogen in *post-mortem* [6] and urokinase-activated [7] human plasma by gel filtration and gel electrophoresis in combination with immunoelectrophoresis using specific antibodies against plasminogen and against the plasma protease inhibitors. He identified plasmin · α_2-macroglobulin complex and a compound, reacting with anti-plasminogen antisera, characterized by early 7-S elution and post-β_1 mobility, which was devoid of plasmin activity, unactivatable by urokinase and which did not react with antisera to the known plasma protease inhibitors. It was concluded that this compound might represent plasmin in a complex with a strong (unknown) inhibitor, a changed and denatured plasminogen or plasmin molecule, or a polymerization phenomenon [7]. It might also be a complex of plasmin with a known protease inhibitor which has lost the antigenic determinants of the inhibitor.

Aoki and von Kaulla [8] and Hedner [9] have identified an α_2-globulin with a molecular weight of approximately 80000 which acted primarily as an inhibitor of plasminogen activation and possessed little or no antiplasmin activity.

Gallimore [10] has recently identified an inter-α-globulin fraction which is a powerful inhibitor of both urokinase-induced and plasmin-induced clot lysis.

In the present work, we have evaluated the relative contribution of the plasma protease inhibitors to the binding of plasmin, generated upon activation of whole plasma with streptokinase or urokinase.

MATERIALS AND METHODS

Plasma

Fresh blood-bank plasma obtained from normal donors, using acid/citrate/dextrose as anticoagulant, was used. Plasma deficient in α_1-antitrypsin was obtained from a patient with congenital α_1-antitrypsin deficiency. The amount of immunoreactive α_1-antitrypsin was 2–5% of normal. Plasma deficient in C_1-esterase inhibitor was obtained from a patient with angioneurotic oedema, having a plasma level which was 18% of normal by immunochemical quantitisation and undetectable by a biological assay. These plasmas were kindly supplied by Dr E. Stevens (University Hospital, Leuven). Normal plasma was depleted of antithrombin III by chromatography on heparin-Sepharose [11], of antiplasmin by chromatography on insolubilized purified antibodies, and of α_2-macroglobulin by gel filtration and recombination of the 7-S and 4-S globulin peaks.

Plasminogen

Human plasminogen was purified by affinity chromatography on lysine-substituted agarose [12], followed by Sephadex G-150 gel filtration and DEAE-Sephadex chromatography [13]. The details of the purification procedure have been described previously [14]. The purified preparations had a specific activity of approximately 25 caseinolytic units/mg (units defined by Committee on Thrombolytic Agents) and less than 0.3% spontaneous proteolytic activity, and were indistinguishable by several physicochemical and biological criteria from unfractioned plasma plasminogen [14]. The two major forms of plasminogen were separated by chromatography on lysine-substituted agarose using a gradient of 6-aminohexanoic acid [15], followed by DEAE-Sephadex chromatography. These two forms of plasminogen both have amino-terminal glutamic acid and a molecular weight of approximately 90000, but a different affinity for insolubilized lysine. They are referred to as plasminogen A1 (first peak) and plasminogen A2 (second peak), and were labelled with ^{125}I and ^{131}I by the method of McFarlane [16] performed as described previously [14].

Plasminogen Activators

Streptokinase (Kabikinase®) was obtained from Kabi AB (Stockholm, Sweden) and urokinase from Abbott (North Chicago, Ill., U.S.A.).

Antisera

Specific rabbit antisera against human α_2-macroglobulin, α_1-antitrypsin, antithrombin III, inter-α-trypsin inhibitor and C_1-esterase inhibitor were purchased from Behringwerke (Marburg/Lahn, West Germany). Antisera against the human plasmin · antiplasmin complex were produced in rabbits with the use of our purified preparations. The protein was dissolved in 0.15 M NaCl to a concentration of 0.2 mg/ml and mixed with an equal volume of Freund's complete adjuvant. 1 ml of this suspension was injected at multiple sites (intracutaneous, subcutaneous and intramuscular). The same procedure, but using Freund's incomplete adjuvant, was repeated at weekly intervals for three weeks. The bleedings were started one week after the last injection. Rabbit antiserum against antiplasmin was obtained by absorption of the serum raised against the complex, with 0.2 mg of purified plasminogen per ml serum. Antisera against human plasminogen were obtained by immunization with the purified material.

Gels

Agarose A5m was obtained from Biorad Laboratories (Richmond, Ca., U.S.A.), Sephadex G-200 from Pharmacia Fine Chemicals (Uppsala, Sweden), Ultrogel AcA44, and agarose for gel electrophoresis from

l'Industrie Biologique Française (Genevilliers, Seine, France). Lysine-substituted agarose was prepared by the CNBr technique as previously described [12], and contained 1–2 g of lysine per 100 ml gel. 6-Aminohexyl-Sepharose was prepared as described by Cambiaso *et al.* [17].

Reagents

Casein was obtained from British Drug House, and further purified as described elsewhere [18]. The synthetic tripeptide D-valyl-leucyl-lysyl-*p*-nitroanilide was a gift from Bofors AB (Mölndal, Sweden).

Electroimmunoassay

Human plasminogen, α_2-macroglobulin, α_1-antitrypsin, antithrombin III, inter-α-trypsin inhibitor, C_1-esterase inhibitor and antiplasmin were determined according to Laurell [19].

Preparation of Antiplasmin-Depleted Plasma

The antiplasmin was removed from fresh human plasma by chromatography on 6-aminohexyl-Sepharose to which specific rabbit immunoglobulins directed against the inhibitor were linked using the method of Cambiaso *et al.* [17]. The immunoglobulin fraction of rabbit antiserum against antiplasmin was precipitated with ammonium sulphate at 40% saturation, and the dialyzed precipitate was chromatographed on the purified plasmin · antiplasmin complex, insolubilized on CNBr-activated Sepharose. The specific immunoglobulins directed against antiplasmin were then eluted from this column with 3 M KSCN. For the present experiments on an analytical scale, we used a 0.9 × 10-cm column of 6-aminohexyl-Sepharose to which the antiplasmin immunoglobulins obtained from 25 ml rabbit serum were coupled. This column could bind the antiplasmin antigen from approximately 10 ml of fresh plasma.

Antiplasmin Assay

Antiplasmin activity was measured with a caseinolytic assay as previously described [18] and with the synthetic substrate D-valyl-leucyl-lysyl-*p*-nitroanilide, using the conditions outlined by the manufacturers but adapted to an end-point method with 2-min reaction time. In the caseinolytic assay, inhibition of plasmin (bovine fibrinolysin, Parke Davis Co., Detroit, Mich.) was measured by preincubation of 1 ml plasmin solution containing an amount of enzymic activity equivalent to that generated upon activation of 20 µl human plasma, with 1 ml of a 50-fold dilution of normal plasma or plasma depleted in antiplasmin, antithrombin III, or α_2-macroglobulin or plasma deficient in α_1-antitrypsin or C_1-esterase inhibitor, for various time intervals (0, 5 min, 10 min and 1 h). In the assay with the synthetic substrate, inhibition of plasmin (human fibrinolysin, Parke Davis Co., Detroit, Mich.) was measured by preincubation of 50 µl of plasmin solution, containing an amount of enzyme activity equivalent to that which can be generated in 0.1 ml human plasma, with 0.1 ml normal or protease-inhibitor-depleted plasma, in a total volume of 0.5 ml for 20 s or 5 min, before addition of the substrate solution.

Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis

This was performed essentially according to Weber and Osborn [20].

RESULTS

Quantitation of the Extent of Plasminogen Activation and Plasmin · Inhibitor Complex Formation by Analytical Gel Filtration

Sephadex G-200 gel filtration of mixtures of normal human plasma with a trace amount of labelled plasminogen revealed a single radioactivity peak, corresponding to the plasma plasminogen peak as determined enzymically and immunologically (Fig. 1A). When trace amounts of ^{125}I-labelled plasminogen A1 and ^{131}I-labelled plasminogen A2 were added to plasma a slightly smaller elution volume was noted for ^{125}I than for ^{131}I (Fig. 2A). Activation to various degrees with urokinase revealed a progressive and parallel disappearance of radioactivity and enzymic activity eluted in the plasminogen position, the ^{125}I disappearing somewhat faster than the ^{131}I. The elution profile of radioactivity in activated samples consistently showed two additional radioactivity peaks, corresponding to the void volume and to an elution volume slightly smaller than that of the globulin peak (Fig. 1 B–D and Fig. 2 B–D). The relative distribution of radioactivity over the two additional peaks varied in the extreme cases from 1:3 to 2:1. In incompletely activated samples, the second peak was predominant over the void volume peak (Fig. 1 B and Fig. 2 B), whereas in completely activated samples the distribution was variable (Fig. 1 D and Fig. 2 D). Gel filtration of mixtures of normal and α_1-antitrypsin-deficient plasma containing a trace amount of labelled plasminogen showed that, after activation with urokinase, the relative distribution of radioactivity over the two additional peaks is independent of the concentration of α_1-antitrypsin in the plasma samples.

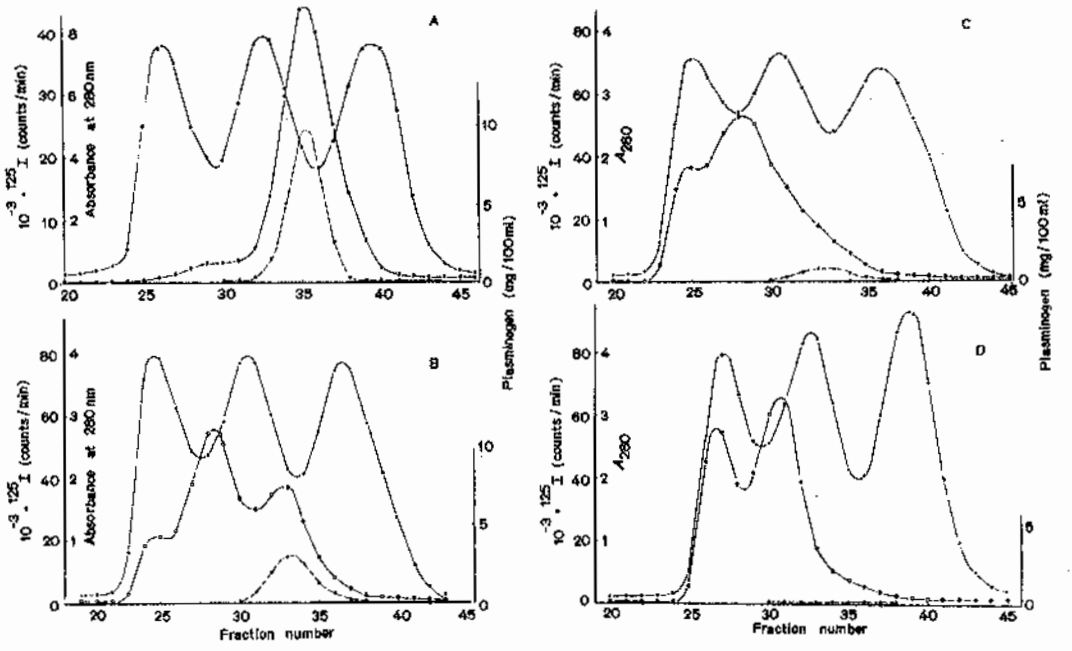

Fig. 1. *Sephadex G-200 gel filtration of a mixture of a trace amount (approximately 5000 counts min^{-1} $ml\ plasma^{-1}$) of ^{125}I-labelled plasminogen with 3 ml human plasma, activated for 30 min at room temperature with increasing amounts of urokinase.* The column (2.5 × 45 cm) was equilibrated with 0.1 M NaCl, 0.1 M 6-aminohexanoic acid, 0.05 M phosphate buffer, pH 7.5 and developed at room temperature at a flow rate of 15–20 ml/h. Urokinase: (A) none, (B) 100 units/ml plasma, (C) 200 units/ml and (D) 500 units/ml. (○——○) ^{125}I; (●——●) absorbance at 280 nm; (●– – –●) plasminogen

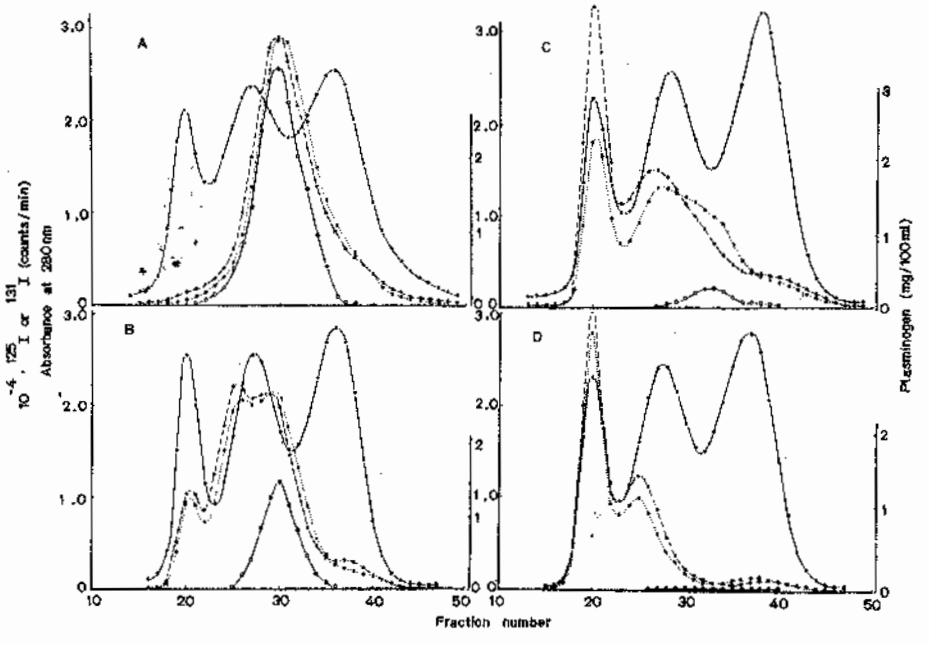

Fig. 2. *Sephadex G-200 gel filtration of a mixture of a trace amount of ^{125}I-labelled plasminogen A1 (first peak from lysine-agarose) and ^{131}I-labelled plasminogen A2 (second peak from lysine-agarose) with 3 ml human plasma, performed as described in the legend of Fig. 1.* (●——●) Absorbance at 280 nm; (●– – –●) ^{125}I; (● · · · · · ●) ^{131}I; (○——○) plasminogen

Purification of the Plasmin · Inhibitor Complexes

Lysine-agarose chromatography of 250 ml of streptokinase-activated human plasma containing trace amounts of ^{125}I-labelled plasminogen A1 and ^{131}I-labelled plasminogen A2 revealed a complex elution pattern for both isotopes (Fig. 3). By gel filtration of several portions of the chromatogram on Sephadex G-200, the following elution sequence from the lysine-agarose column could be established: (a) the

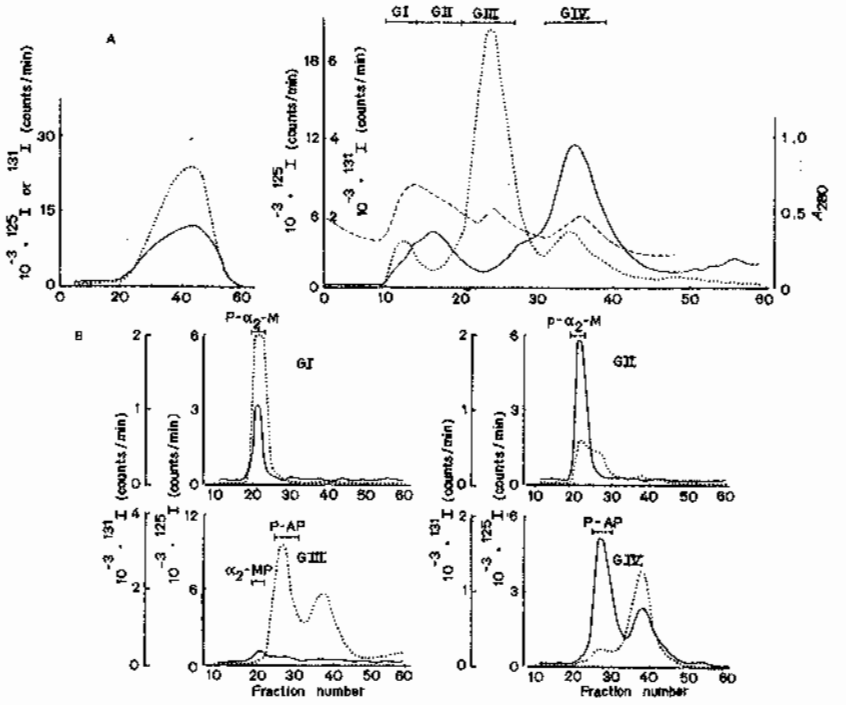

Fig. 3. *Purification of plasmin · inhibitor complexes.* (A) Lysine-agarose chromatography of human plasma containing trace amounts of ^{125}I-labelled plasminogen A1 and ^{131}I-labelled plasminogen A2, activated with 250–500 units of streptokinase per ml plasma. The 2.5 × 45-cm column was equilibrated with 0.1 M phosphate buffer pH 7.5. The plasma was passed through the column at a flow rate of 50–70 ml/h and non-adsorbed protein removed by washing with the equilibration buffer. Elution was performed with a linear gradient consisting of 500 ml 0.1 M phosphate pH 7.5 as starting buffer and 500 ml 0.1 M phosphate containing 0.013 M 6-aminohexanoic acid as limiting buffer. The eluates were pooled as indicated and concentrated by vacuum dialysis or ultrafiltration. Left part: non-adsorbed radioactivity, usually approximately 10% and consisting mainly of plasmin · α_2-macroglobulin complex. Right part: sequential elution of plasmin · α_2-macroglobulin (P-α_2-M), plasmin · antiplasmin (P-AP) and plasminogen peaks. (———) ^{131}I; (·····) ^{125}I; (–·–·–) absorbance at 280 nm. (B) Sephadex G-200 gel filtration of the eluates from the lysine-agarose columns, pooled as indicated in (A)

complex of plasminogen A1 eluted at the void volume, (b) the complex of plasminogen A2 eluted at the void volume, (c) the complex of plasminogen A1 eluted just before the globulin peak, (d, e) the complex of plasminogen A2 eluted just before the globulin peak, coinciding with residual plasminogen A1, and (f) residual plasminogen A2.

The pooled fractions of the various Sephadex G-200 columns were finally collected in three pools. The first pool, termed P-α_2 M, comprised the protein containing ^{125}I and ^{131}I eluted at the void volume and corresponding to the first radioactive peak eluted on the analytical gel filtration column. The second pool, termed P–AP, contained the protein containing ^{125}I and ^{131}I eluted in the position corresponding to the second peak of the analytical gel filtration column. The third pool, corresponding to the elution position of plasminogen represented residual small amounts of ^{125}I-labelled plasminogen A1 and ^{131}I-labelled plasminogen A2. These pools were dialyzed against distilled water and lyophilized. The average recovery from four runs of 2000 ml plasma each was 14.5 A_{280} units in the first peak and 7.3 A_{280} units in the second peak, per 100 ml plasma.

Characterization of the Plasmin · Inhibitor Complexes

The purified materials corresponding to the first (P-α_2 M) and second (P-AP) radioactive peaks were characterized by dodecylsulphate polyacrylamide gel electrophoresis and immunoelectrophoresis in gels containing specific antisera. Dodecylsulphate polyacrylamide gel electrophoresis (Fig. 4) of the material eluted in the first peak (P-α_2 M) showed a band which barely entered a 7% gel (M_r > 400000). In the presence of dithioerythritol, several bands were observed including a main one with M_r 95000 and a faint one with M_r approximately 65000. The material eluted in the second peak (P-AP) showed two main bands with estimated M_r of approximately 120000 and 140000. Dodecylsulphate-polyacrylamide gel electrophoresis in the presence of 8 M urea revealed only one band with an estimated M_r of approximately 130000. In the presence of dithioerythritol, two closely migrating bands were observed with M_r of approximately 65000–70000 and one band with M_r 11000.

Immunoelectrophoresis in agarose gel containing antisera against human plasminogen, α_2-macroglobulin, α_1-antitrypsin, antithrombin III, inter-α-trypsin

inhibitor, or C_1-esterase inhibitor showed precipitin formation of the P-α_2 M peak material only with anti-α_2-macroglobulin and a weak precipitin formation with anti-plasminogen. The P-AP peak material reacted only with anti-plasminogen and not with antisera against the known protease inhibitors. Immunoelectrophoresis in gels containing antisera against the purified P-AP peak material and absorbed with purified plasminogen (antiserum to antiplasmin) revealed a precipitin line both with the purified complex and with plasma, but not with the purified P-α_2 M peak material. Crossed immunoelectrophoresis of fresh or urokinase-activated plasma using mixtures of antisera against antiplasmin and against the known plasma protease inhibitors, have indicated that antiplasmin is a different entity to α_1-antitrypsin, α_2-macroglobulin, C_1-esterase inhibitor, antithrombin III, and inter-α-trypsin inhibitor. These experiments have been reported elsewhere [21]. No proteolytic activity could be demonstrated by a 100-fold molar excess of the plasmin · inhibitor complex as compared to the amount of plasmin used in the caseinolytic or the synthetic substrate assays.

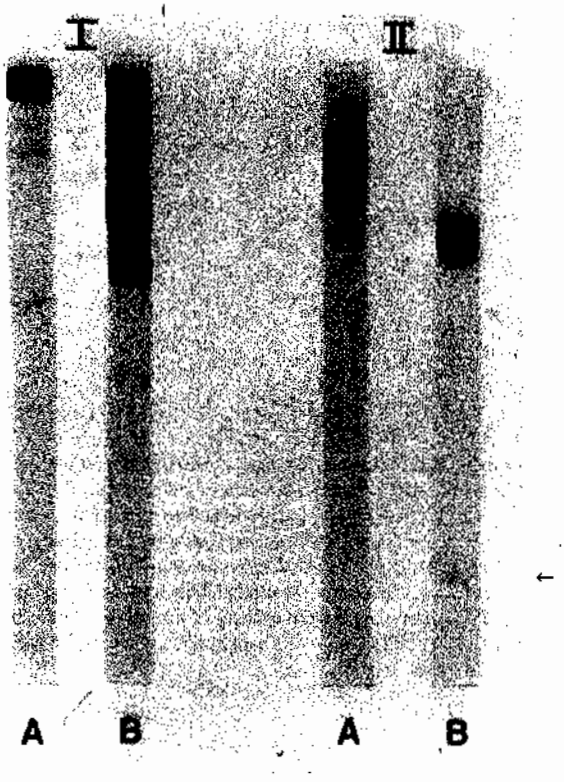

Fig. 4. *Dodecylsulphate-polyacrylamide gel electrophoresis.* (I) Plasmin · α_2-macroglobulin complex; (II) plasmin · antiplasmin complex. (A) No dithioerythritol; (B) in the presence of dithioerythritol

Inhibition of Plasmin by Normal Plasma and by Plasma Deficient in the Protease Inhibitors α_1-Antitrypsin, C_1-Esterase Inhibitor, Antithrombin III, Antiplasmin and α_2-Macroglobulin

The relative contribution of the plasma protease inhibitors to the binding of plasmin was investigated by comparison of the inhibitory activity of plasma deficient in one of the protease inhibitors with that of whole plasma. The deficient or depleted plasmas were obtained as described under Materials and Methods. The specific absence of a single protease inhibitor in each of these plasmas was checked by immunoelectrophoresis in agarose gel containing specific antibodies.

Table 1 summarizes the data on the inhibition of plasmin by normal and specifically deficient human

Table 1. *Inhibition of plasmin by normal human plasma and by plasmas depleted in protease inhibitors*
Antiplasmin concentration was determined by electroimmunodiffusion in agarose gel containing antiplasmin antibodies; it is expressed as a percentage of that of a pool of 20 normal plasmas. The percentage of plasmin inhibited was assayed both by the synthetic tripeptide method and by the caseinolytic assay, as decribed in Materials and Methods. Results are given as the mean ± S.D. with the number of experiments in parentheses

Plasma	Antiplasmin concn	Plasmin inhibited			
		tripeptide assay		caseinolytic assay	
		fast (20 s)	total (5 min)	fast (0)	total (10 min)
	%	%			
Normal	100	45 ± 6	65 ± 8 (5)	59 ± 7	87 ± 2 (7)
Antiplasmin-depleted	0	8 ± 3	30 ± 5 (5)	26 ± 5	78 ± 3 (5)
Antithrombin-III-depleted	78	47 ± 7	63 ± 8 (4)	48 ± 7	85 ± 2 (4)
α_1-Antitrypsin-deficient	100	70 ± 22[a]	81 ± 19 (3)	58 ± 4	86 ± 1 (2)
C_1-Esterase-inhibitor-deficient	74	43 ± 4	54 ± 4 (2)	54 ± 2	86 ± 1 (2)
α_2-Macroglobulin-depleted	65	23 ± 3	50 ± 5 (4)	31 ± 7	55 ± 4 (4)
α_2-Macroglobulin peak[b]	–	2 ± 4	15 ± 6 (4)	10 ± 8	50 ± 16 (4)

[a] The individual results are 96, 60 and 55%.
[b] The 19-S peak of AcA44 gel filtration reconcentrated to the original plasma volume.

plasma using both the synthetic substrate and the caseinolytic assay. In normal plasma, the fast antiplasmin has neutralized 45 ± 6% of the added plasmin as determined with the synthetic substrate (2-min reaction time) and 59 ± 7% as determined by caseinolysis (1-h reaction time). This fast-reacting inhibitor activity is decreased to 8 ± 3% and 26 ± 5% in the antiplasmin-depleted plasma, but is essentially normal in plasma depleted in the plasma protease inhibitors antithrombin III, C_1-esterase inhibitor or α_1-antitrypsin. It is possible that some of the residual inhibitor activity thus measured results from neutralization of plasmin by the slower reacting inhibitor(s) during the reaction with the substrate. Although plasma depleted in α_2-macroglobulin showed mainly a decrease of fast-reacting antiplasmin, control experiments with the reconcentrated α_2-macroglobulin-containing 19-S fraction indicated that α_2-macroglobulin reacted slowly with plasmin as determined in the casein assay but did not significantly neutralize the amidolytic activity of plasmin. These data can probably be explained from the well-known fact that the plasmin · α_2-macroglobulin has esterase (or amidase) but no protease activity. These data taken together with our findings on the distribution of plasmin between α_2-macroglobulin and antiplasmin (Fig. 1 and 2) indicate that antiplasmin is a faster-reacting plasmin inactivator than α_2-macroglobulin.

DISCUSSION

In the present study, the relative contribution of the plasma protease inhibitors to the binding of plasmin, generated upon activation of the plasma fibrinolytic system (with streptokinase or urokinase), was investigated. Two plasmin : inhibitor complexes, purified with high yield by affinity chromatography on lysine-agarose and gel filtration, proved to be plasmin · α_2-macroglobulin and a complex between plasmin and a plasma protein which, on the basis of immunochemical studies, turned out to be different from the known protease inhibitors. We have provisonally named this inhibitor antiplasmin; it appears to be a hitherto unidentified plasma protein.

The purified plasmin · antiplasmin complex displayed a doublet band on dodecylsulphate polyacrylamide gel electrophoresis with estimated molecular weight in the range of 120000–140000. After reduction of the disulfide bonds a doublet band was obtained with molecular weight of 65000–70000 and a band of 11000. No band was seen migrating in the position of the plasmin light chain. In analogy with the mechanism of inhibition of trypsin by pancreatic trypsin inhibitor or soy-bean trypsin inhibitor [22], or that of thrombin by antithrombin III [23] these findings are consistent with the hypothesis that the plasmin · antiplasmin complex consists of a covalent bond between the catalytic serine residue of the enzyme and a specific lysine or arginine residue of the reactive site of the inhibitor. The dodecyl sulphate-polyacrylamide gel pattern of the reduced plasmin · antiplasmin complex would thus display the plasmin heavy chain (M_r 65000), the plasmin light chain (M_r 25000) linked covalently to the NH_2-terminal part of the inhibitor (total M_r 65000–70000), and the COOH-terminal part of the inhibitor (M_r 11000). The molecular weight of the inhibitor would thus be between 50000 and 60000.

The different identity of antiplasmin from the known plasma protease inhibitors is indicated by the following findings. (a) The purified complex does not react with antisera against the known plasma protease inhibitors but reacts with antisera raised against the complex and absorbed with plasminogen. (b) This absorbed antisera reacts with a component in plasma which is different from the known plasma protease inhibitors [21]. (c) Specific removal of this protein by absorption with insolubilized antibodies virtually completely eliminates the fast-reacting antiplasmin activity of plasma, without altering its content of the other plasma protease inhibitors.

Further studies on the role of antiplasmin indicated that it is the main and the fastest-reacting plasmin inhibitor. When the plasma plasminogen is activated, the initially formed plasmin binds predominantly to this inhibitor. Apparently it is only upon complete activation of the plasminogen, leading to a relative exhaustion of the antiplasmin, that the α_2-macroglobulin comes into play. Our studies on the addition of plasmin to normal and protease-inhibitor-depleted plasma confirmed that antiplasmin is the fastest-reacting inhibitor and that, under the experimental conditions used, α_1-antitrypsin, antithrombin III and C_1-esterase inhibitor do not participate in the binding of plasmin. These findings necessitate a revision of the current view on the identity of the plasma plasmin inhibitors. Since the work of Norman [2] it has generally been accepted that there are essentially two functionally important plamin inhibitors in plasma, a rapidly reacting plasmin inhibitor, subsequently identified as α_2-macroglobulin, and a slow-reacting inhibitor with α_1 mobility on electrophoresis. Since the work of Rimon *et al.* [4] and of Schwick *et al.* [5] it has generally been accepted that the slow plasmin inhibitor is identical with α_1-antitrypsin. The importance of the other plasma protease inhibitors, C_1-esterase inhibitor, inter-α-trypsin inhibitor and antithrombin III (and especially the antithrombin-III · heparin complex) is still under debate. Our studies show that only two plasma proteins are functionally important in the binding of plasmin generated by activation of the plasma plasminogen (with either streptokinase or urokinase), namely antiplasmin, which is the fastest-reacting inhibitor, and α_2-macroglobulin, the role of

which seems to be to neutralize the remaining plasmin. In the presence of normal plasma levels of these proteins, the other protease inhibitors do not participate significantly in the inactivation of plasmin.

The relationship between antiplasmin and the substances and activities described by other authors requires some comments.

The compound identified by Müllertz [7], named $7S_1$-postβ_1 by him, is most probably identical with our plasmin · antiplasmin complex. Recently, Müllertz has compared our antiserum against antiplasmin, with antisera raised against his compound and absorbed with plasminogen, and found that both sera reacted with the same component in plasma (personal communication). From comparative experiments with our antisera against antiplasmin with antisera prepared by Hedner against the inhibitor of plasminogen activation it could be clearly concluded that these two antisera react with different entities in plasma [24].

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