Biochem. J. (1976) **159**, 545–553
Printed in Great Britain

The Primary Inhibitor of Plasmin in Human Plasma

By STEN MÜLLERTZ and INGE CLEMMENSEN
Department of Clinical Chemistry, University of Copenhagen, Hvidovre Hospital, 2650 Hvidovre, Denmark

(*Received* 19 *May* 1976)

A complex between plasmin and an inhibitor was isolated by affinity chromatography from urokinase-activated human plasma. The complex did not react with antibodies against any of the known proteinase inhibitors in plasma. A rabbit antiserum against the complex was produced. It contained antibodies against plasminogen+plasmin and an α_2 protein. By crossed immunoelectrophoresis the α_2 protein was shown to form a complex with plasmin, when generated by urokinase in plasma, and with purified plasmin. The α_2 protein was eluted by Sephadex G-200 gel filtration with K_D approx. 0.35, different from the other inhibitors of plasmin in plasma, and corresponding to an apparent relative molecular mass (M_r) of about 75000. By sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the M_r of the complex was found to be approx. 130000. After reduction of the complex two main bands of protein were observed, with M_r about 72000 and 66000, probably representing an acyl-enzyme complex of plasmin–light chain and inhibitor–heavy chain, and a plasmin–heavy chain. A weak band with M_r 9000 was possibly an inhibitor–light chain. The inhibitor was partially purified and used to titrate purified plasmin of known active-site concentration. The inhibitor bound plasmin rapidly and strongly. Assuming an equimolar combining ratio, the concentration of active inhibitor in normal human plasma was estimated to be 1.1 μmol/l. A fraction about 0.3 of the antigenic inhibitor protein appeared to be functionally inactive. In plasma, plasmin is primarily bound to the inhibitor. Only after its saturation does lysis of fibrinogen and fibrin occur and a complex between plasmin and α_2 macroglobulin appear.

The serine proteinase plasmin (EC 3.4.21.7) is formed from a precursor, plasminogen, present in mammalian blood plasma. The physiological function of plasmin is the degradation and dissolution of fibrin deposits in the vessels. Plasma contains several proteinase inhibitors, which in purified form inhibit purified plasmin (Heimburger *et al.*, 1971), but α_2 macroglobulin is generally considered to be the only inhibitor of physiological importance (Niléhn & Ganrot, 1967). However, besides a plasmin–α_2 macroglobulin complex, another compound with plasminogen antigenicity was demonstrated in fibrinolytic post-mortem plasma (Müllertz, 1972) and in plasma after addition of urokinase (EC 3.4.99.26), a biological activator of plasminogen (Müllertz, 1974). The compound was eluted earlier from Sephadex G-200 (K_D approx. 0.18) than was plasminogen (K_D approx. 0.35), had a different (post-β_1) mobility on agarose-gel electrophoresis, was not dissociable and had no enzymic or proenzymic activity. The compound was probably a plasmin–inhibitor complex, but the various well-known proteinase inhibitors in plasma were ruled out by immunological and protein-separating methods. Thus the data indicated the existence in plasma of an unknown inhibitor with a higher avidity than that of α_2 macroglobulin for plasmin. Collen (1974) briefly reported the existence of two plasmin–inhibitor complexes in human plasma, a plasmin–α_2 macroglobulin complex and a plasmin–α_1 anti-plasmin complex. Collen *et al.* (1975) presented immunochemical evidence that human plasma contains an α_2 protein that forms a complex with plasminogen antigenicity in plasma after activation by streptokinase and urokinase. The inhibitor was different from the known proteinase inhibitors in plasma.

The object of the present paper is to describe the isolation and some properties of the plasmin–inhibitor complex from urokinase-activated human plasma, and to demonstrate the presence and some properties of a primary plasmin inhibitor in human plasma different from previously known inhibitors.

Experimental

Materials

Buffers for gel electrophoresis and gel filtration were as follows. Tris buffer: Tris/HCl, 0.1 mol/l; NaCl, 0.05 mol/l; NaN_3 3 mmol/l; NaOH, 1 mol/l, added to pH 7.6; $I = 0.15$ mol/l. Tris/lysine buffer: Tris/HCl, 0.05 mol/l; lysine hydrochloride, 0.05 mol/l; NaCl, 0.05 mol/l; NaN_3, 3 mmol/l; NaOH, 1 mol/l, added to pH 7.6; $I = 0.15$ mol/l. Tris/barbiturate buffer: Tris, 74mmol/l; diethylbarbituric acid, 25mmol/l; calcium lactate, 0.3mmol/l, NaN_3, 2mmol/l, pH 8.6, $I = 0.07$ mol/l. Human citrated plasma was obtained from donor blood stabilized by 0.13 vol. of sodium citrate (0.73 mol/l)/citric acid (38 mmol/l)/glucose (0.124 mol/l). Protein standard plasma with known concentrations of the relevant plasma proteins was from Behringwerke, Marburg-Lahn, West Germany.

The following materials were described in Müllertz (1974): urokinase, normal human plasma, specific rabbit immunoglobulins against α_1 anti-trypsin, α_2 macroglobulin, human serum protein, inter-α-inhibitor, C_1-esterase inhibitor, anti-thrombin III and antiserum against plasminogen.

Immunoglobulin against anti-chymotrypsin, transferrin and albumin were obtained from Dakopatts, Copenhagen, Denmark. A sample of adsorbed rabbit immunoglobulin against a plasma inhibitor (Collen *et al.*, 1975) was kindly given by Dr. D. Collen, Department of Medical Research, University of Leuven, Leuven, Belgium.

DEAE-Sephadex A-50, concanavalin A–Sepharose and columns for chromatography were from Pharmacia Fine Chemicals, Uppsala, Sweden. Ultrogel ACA 34 was from LKB, Stockholm, Sweden.

Lysine–Sepharose was prepared as described by Deutsch & Mertz (1970). Urokinase–Sepharose was prepared by coupling urokinase to Sepharose 4B by the CNBr technique of Axén *et al.* (1967) essentially as described by Wiman & Wallén (1973). The final preparation contained 4×10^7 Ploug units/l (approx. 9.5 μmol/l).

Methods

Immunochemical techniques. The concentration of the plasma proteinase inhibitors and plasminogen+plasmin was determined by quantitative electroimmunoassay in agarose gel containing specific antibodies, as described by Laurell (1972). Duplicate determinations were done. Plasminogen-adsorbed anti-complex immune serum was used for the assay of the primary plasmin inhibitor. Crossed immunoelectrophoresis was performed in agarose gel, essentially as described by Ganrot (1972). Identification of protein bands in polyacrylamide gels was done by washing the tube gels in Tris buffer for 72h and moulding the gels into agarose gel with antibodies on a glass plate and performing a second-dimension electrophoresis at a potential gradient of 1 V/mm for 17h. Extensive washing was necessary to avoid unspecific precipitation of protein by sodium dodecyl sulphate in the second step. Tris/barbiturate buffer, pH 8.6, was used for the second-dimension electrophoresis. The concentration of antigenic protein was calculated from the area of the precipitates obtained by quantitative crossed immunoelectrophoresis as described by Weeke (1973). Ouchterlony gel immunodiffusion was performed as described by Clausen (1969).

Analytical and preparative gel filtration. This was performed with Sephadex G-200 as described by Müllertz (1972, 1974); Tris buffer, pH 7.6, was used.

Affinity chromatography on lysine–Sepharose. For plasminogen and plasmin complexes this was a batch modification (Rickli & Cuendet, 1971) of the original procedure (Deutsch & Mertz, 1970). The plasmin–inhibitor complex was bound specifically to Sepharose-linked lysine.

For plasminogen, 700 ml of human serum was diluted with an equal volume of buffer (sodium phosphate, 0.1 mol/l, pH 7.4) and stirred with 100g of lysine–Sepharose and 100g of Sepharose for 30min at 4°C. For the plasmin–inhibitor complex, the serum was replaced by the pooled fractions (about 300ml) from a Sephadex G-200 gel filtration. The lysine–Sepharose with adsorbed protein was washed on a Buchner funnel with sodium phosphate buffer (0.3 mol/l), pH 7.4, until the E_{280} of the eluate was below 0.01. The washed material was suspended in 200ml of the same buffer and allowed to settle in a column (Pharmacia K 25/45; 0.45 m × 494 mm²). The plasminogen or plasmin complex was eluted with buffer [6-aminohexanoic acid (0.2 mol/l)/sodium phosphate (0.1 mol/l), pH 7.4] at a rate of 100 ml/h. The eluate was monitored by photometry. All fractions with an E_{280} higher than 0.01 were pooled and desalted on a column of Sephadex G-25 eluted with Tris buffer at 4°C at a rate of 15 ml/h. The eluate was concentrated by ultrafiltration (see below), and stored at −20°C.

Estimation of protein concentration. An approximate estimate was obtained from the E_{280}, by using a specific absorbance coefficient of $100\,m^2 \cdot kg^{-1}$.

Assay of enzyme activity. Benzoylarginine ethyl esterase activity was determined as described by Müllertz (1974). Fibrin-clot lysis was assayed as described by Thorsen (1973).

Ultrafiltration. This was done in a stirred cell (Amicon, Lexington, MA, U.S.A.), model 202 or 52 with a PM 10 membrane, which retained molecules with M_r higher than 10000.

Active-site titration. This was done with *p*-nitrophenyl-*p'*-guanidinobenzoate essentially as described by Chase & Shaw (1969) with a Beckman spectrophotometer model 25 and microcuvettes.

Assay of the apparent relative molecular mass (M_r) by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The technique was that described by McDonagh *et al.* (1972), except that the gel contained acrylamide (100g/l) and methylenebisacrylamide (1.35 g/l). A constant current of 14mA per tube (cross-sectional area 12.6mm²) was applied for 3h. A Gradipore instrument from Gradient Pty Ltd., Lane Cove, N.S.W., Australia was used. α-Chymotrypsin (M_r 27500), ovalbumin (M_r 45000), lysozyme (M_r 14400) (from Sigma, St. Louis, MO, U.S.A.), transferrin (M_r 74000) (from Behringwerke), phosphorylase *a* (M_r 94000) (from Boehringer, Mannheim, West Germany), and γ-globulin (IgG) (M_r 150000) (from Kabi, Stockholm, Sweden) were used as reference markers. The sample was run both separately and together with the reference proteins in a single tube.

Assay of apparent M_r by gel filtration. This was done as described by Andrews (1965) by applying K_D values obtained by Sephadex G-200 gel filtration of normal plasma for the following plasma proteins: C_1-esterase inhibitor (M_r 104000, K_D 0.17), plasminogen (M_r 85000, K_D 0.35), transferrin (M_r 74000, K_D 0.39), albumin (M_r 69000, K_D 0.42), anti-chymotrypsin (M_r 69000, K_D 0.35), anti-thrombin III (M_r 62000, K_D 0.41), α_1 anti-trypsin (M_r 61000, K_D 0.42). The K_D values were from the experiment shown in Fig. 1. The regression of K_D (=y) on $\log M_r$ (=x) was calculated according to the principle of least squares. The function was $y = 5.159 - 0.818x$. The coefficient of correlation was 0.91 and $s_{y/x} = 0.037$.

Preparation of plasminogen. Plasminogen was prepared in its native form from human plasma by affinity chromatography (see above). The content of protein was determined by weighing after freeze-drying and correction for salt content. The content of activatable plasminogen was determined by active-site titration after maximal activation in glycerol with urokinase as described previously (Müllertz, 1974). The mass concentration of protein was 6.6 g/l and the concentration of activatable protein was 60 μmol/l which is approx. 5.1 g/l (M_r of plasminogen 85000).

Preparation of plasmin. Plasmin was prepared in an activator-free state, in principle as described by Wiman & Wallén (1973). A small glass column (bed dimensions 6.5 mm × 71 mm^2) was filled with a suspension of urokinase–Sepharose (460 μl containing 18400 Ploug units of urokinase, which is approx. 4.4 nmol). The plasminogen solution was aspirated and pumped through the column, at a flow rate of 10 μl/s, by means of an LKB Varioperpex peristaltic pump through Teflon tubes with an internal diameter of 0.1 mm, connected to the inlet and outlet of the column. The eluate was collected in glycerol (final volume fraction 0.5). The active-site concentration of plasmin and of activatable plasminogen+plasmin in the pooled eluate was 3.8 and 4.7 μmol/l respectively.

Isolation of a plasmin–inhibitor complex. Human citrated plasma (160 ml) was activated by urokinase (3500 Ploug units) for 30 min at 37°C, and subjected to preparative Sephadex G-200 gel filtration. The fractions eluted with the early 7 S fraction proteins were pooled as previously described (Müllertz, 1974). The pool contained a complex with plasminogen antigenicity, which had post-β_1 mobility on agarose-gel electrophoresis at pH 8.6, but no other components or complexes with plasminogen antigenicity. The pooled fractions were subjected to affinity chromatography on lysine–Sepharose (see above) and the final eluate was concentrated by ultrafiltration to a final volume of 9 ml. The mass of the complex recovered from 160 ml of urokinase-activated citrated plasma was about 3, 4 and 7 mg (three preparations).

Production of an immune serum against the plasmin–inhibitor complex. The preparation of the purified complex with a protein content of approx. 0.3 g/l was homogenized with an equal volume of Freund's complete adjuvant plus 2 vol. of NaCl (0.15 mol/l), and a dose of 400 μl was injected subcutaneously in four places in a rabbit with intervals of 14 days between each injection. After 1–2 months after the last injection the rabbit serum had reached a satisfactory titre of antibodies against the complex. In both sera, weak antibodies against three to four other proteins were detectable, in one serum in very low titres, however. The latter antibodies were totally removed by adsorption with a concentrated pool of protein prepared from the fractions that were eluted earlier than native plasminogen and the primary plasmin inhibitor by Sephadex G-200 gel filtration of normal plasma. Between 0 and 3 mg of protein was added per ml of rabbit serum. The serum was left overnight at 5°C, and the precipitate was discarded. Adsorption with plasminogen was done in the same way, by adding plasminogen (27 μmol/l) to rabbit serum at a volume fraction of 0.1.

A crude preparation of the primary plasmin inhibitor. The fractions from Sephadex G-200 preparative gel filtration eluted with K_D values between 0.28 and 0.35 (see Fig. 1) were pooled and concentrated by ultrafiltration. The mass concentration of the inhibitors in the preparation with an effect on plasmin was determined by electroimmunoassay by reference to a protein standard plasma and the substance concentrations were calculated by means of the relative molecular masses (M_r) (see above). The following values were found: α_1 anti-trypsin, 1.3 μmol/l; antithrombin III, 0.6 μmol/l; C_1 esterase inhibitor, 1.3 μmol/l; α_2 macroglobulin (M_r 725000), 0.2 μmol/l.

Partial purification of the primary inhibitor of plasmin. The probable nature of the reaction between the inhibitor and plasmin precluded the isolation of the inhibitor in its native form by dissociation of the isolated plasmin–inhibitor complex. The purification was performed by conventional methods of protein fractionation. The steps were monitored by electroimmunoassay of the antigenic α_2 protein and of the known inhibitors and by a fibrin-clot-lysis assay. The inhibition of urokinase-induced fibrinolysis covaried with the concentration of the plasmin inhibitor. The concentration of inhibitor at each step could not be determined by titration of plasmin in the presence of large amounts of other inhibitors, but was calculated from the concentration of antigenic protein with reference to the crude inhibitor preparation, assuming the same ratio of antigenic protein to active inhibitor in all materials (see the Results section). The value for the final preparation was obtained by titration. The specific amount of inhibitor (μmol/g) was calculated from the substance concentration of inhibitor divided by the mass concentration of protein. The recovered fractions were calculated from the amount of antigenic protein at each step.

Step 1. Fractionation by $(NH_4)_2SO_4$. Human citrated plasma (360ml) with a specific amount of inhibitor of 0.015μmol/g was processed. Solid $(NH_4)_2SO_4$ was added slowly, while stirring, to the plasma to 0.8mol/l, calculated from nomograms of mass/volume per mass concn. per saturation. After 1h the precipitate was separated by centrifugation for 30min at 4°C at 5000g, and the concentration of $(NH_4)_2SO_4$ in the supernatant was increased to 2.7mol/l. After 1h the precipitate was collected as described, dissolved in water (150ml) and dialysed against tap water at 4°C overnight and against sodium acetate buffer (0.05mol/l), pH6.0, for 24h. This step removed plasminogen, fibrinogen and most α_2 macroglobulin and α_1 anti-trypsin. The specific amount was 0.018μmol/g and the recovered fraction 0.65.

Step 2. Ion-exchange chromatography. DEAE-Sephadex A-50 in a column ($0.45\,m \times 2.04 \times 10^3\,mm^2$) was equilibrated at 4°C with sodium acetate buffer (0.05mol/l)/NaCl (0.06mol/l), pH6.0; I was 0.11mol/l. The non-diffusible material (350ml) was applied, washed with 1 litre of the buffer and eluted by a linear salt gradient prepared with 1 litre of the buffer and 1 litre of sodium acetate buffer (0.05mol/l)/NaCl (0.5mol/l), I 0.55mol/l. This step removed the remaining α_1 anti-trypsin, anti-thrombin III and other proteins including lipoproteins. The inhibitor was eluted between $I = 0.27$ and 0.31mol/l. The specific amount was 0.040μmol/g and the recovered fraction was 0.40.

Step 3. Isolation of glycoproteins. Concanavalin A–Sepharose in a column ($0.45\,m \times 510\,mm^2$) was equilibrated at 4°C with Tris/HCl buffer (0.05mol/l)/$MgCl_2$ (1mmol/l)/$MnCl_2$ (1mmol/l)/$CaCl_2$ (1mmol/l) pH7.7 (20°C), I0.05mol/l. To the pooled fractions with inhibitor from Step 2 (190ml) were added $MgCl_2$, $MnCl_2$ and $CaCl_2$ to the same concentrations as in the buffer; the mixture was applied. Unadsorbed protein was eluted by percolating 1 litre of the buffer through the column. The glycoproteins were eluted with the same buffer containing methyl α-D-glucopyranoside (0.1mol/l; 400ml) and concentrated by ultrafiltration to 25ml. This step removed albumin and retained the inhibitor and anti-chymotrypsin. The specific amount was 0.06μmol/g and the recovered fraction was 0.33.

Step 4. Gel filtration. Ultrogel ACA 34 in a column ($1\,m \times 510\,mm^2$) was equilibrated at 4°C with Tris/HCl buffer (0.05mol/l; pH8.6; I0.05mol/l). The glycoprotein pool was applied and eluted with the buffer. The fractions with inhibitor between K_D0.32 and 0.36 were pooled. This step removed macromolecules and part of anti-chymotrypsin. The specific amount was now 0.34μmol/g and the recovered fraction 0.22.

Step 5. Ion-exchange chromatography. DEAE-Sephadex A-50 in a column (bed volume $0.2\,m \times 510\,mm^2$) was equilibrated at 4°C with the buffer of Step 4. The pooled fractions with inhibitor from Step 4 were applied, washed with the buffer (200ml) and eluted by a linear salt gradient prepared from the buffer (300ml) and Tris/HCl buffer (0.05mol/l)/NaCl (0.5mol/l), pH8.6. The fractions between $I = 0.25$ and 0.28mol/l were pooled and concentrated by ultrafiltration to 5ml. This step removed anti-chymotrypsin. The specific amount of inhibitor was 1.56μmol/g and the recovered fraction 0.10. The concentration of active inhibitor was 3.90μmol/l (see the Results section). The preparation contained no detectable amounts of known proteinase inhibitors, as determined by electroimmunoassay against their specific antibodies.

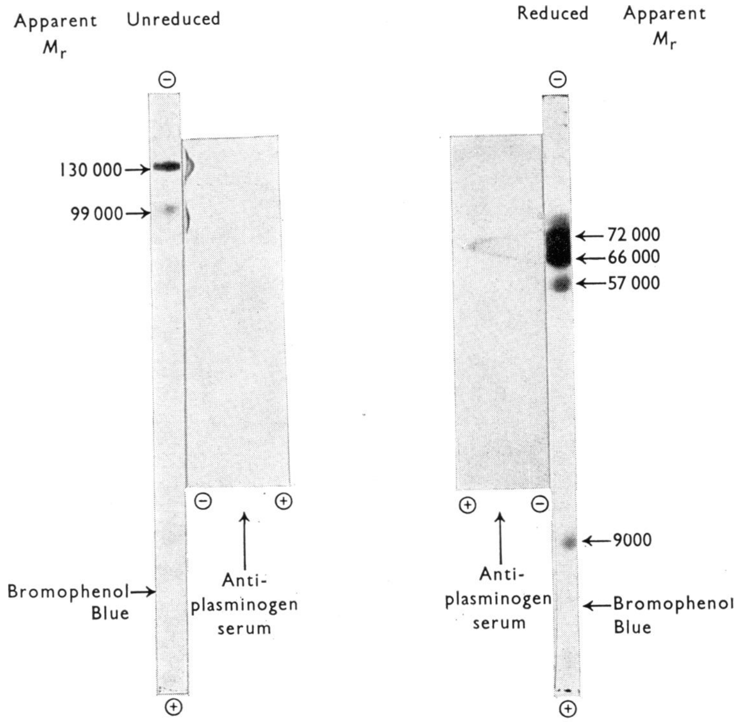
The mass fraction (active + inactive inhibitor)/(total protein) was 0.0016 for the plasma used and 0.3 for the final preparation on the pre-supposition that 0.7 and 0.4 of the antigenic protein of the two materials respectively were functionally active and that a M_r of 75000 was applied (see the Results section).

Results

Properties of the plasmin–inhibitor complex

The material with the isolated plasmin–inhibitor complex was studied by crossed immunoelectrophoresis in agarose gel, containing antibodies against human serum proteins or specific antibodies against one of the following proteins: plasminogen, α_1 anti-trypsin, anti-thrombin III, inter-α-inhibitor, C_1-esterase inhibitor and α_2 macroglobulin, and anti-chymotrypsin. No precipitates were detectable in the gel containing antibodies against the inhibitors, but a single large homogeneous and distinct precipitate with post-β_1 mobility was formed in the gel with antibodies against human serum proteins or with anti-plasminogen serum. Hence the material contained very little contaminating protein.

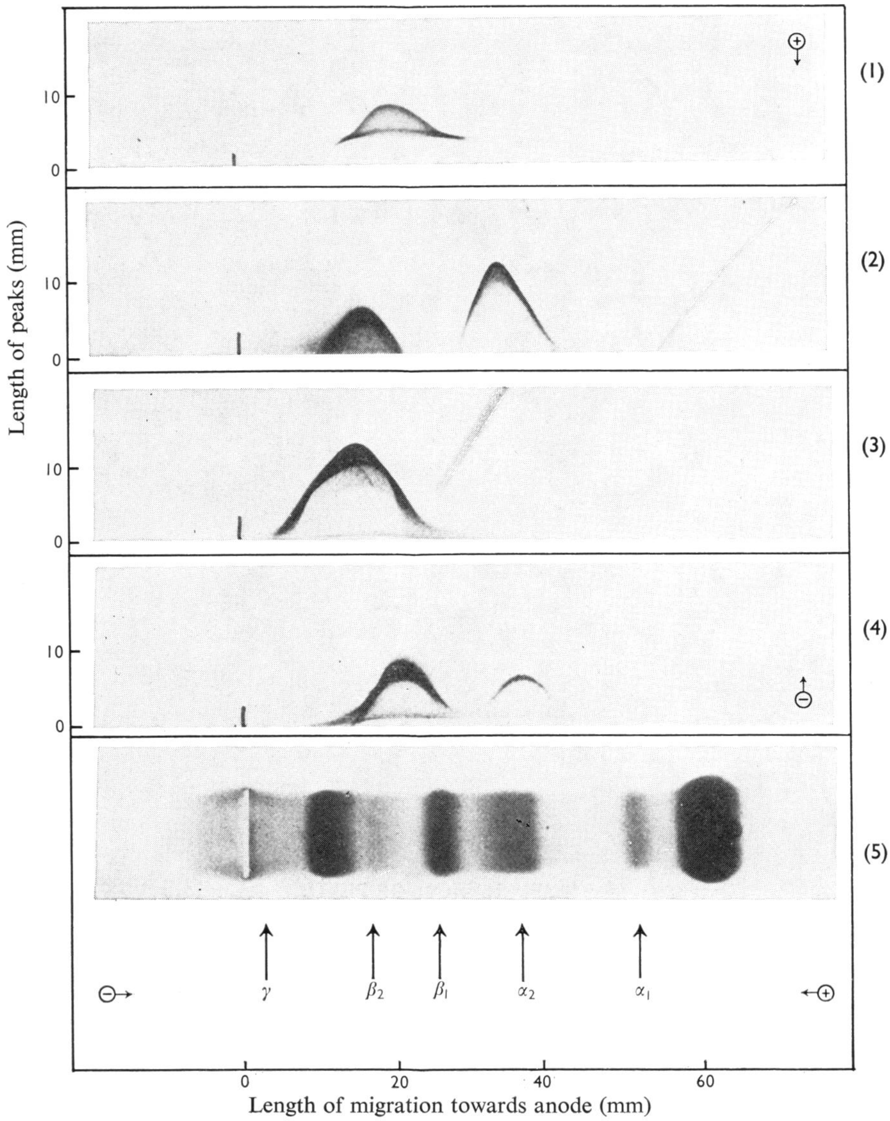
The material (three preparations) was studied by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Plate 1). The apparent relative molecular masses (M_r) of the bands were calculated by reference to the migration of marker proteins with known M_r. Without reduction, a distinct band with M_r approx. 130000 and a faint band with M_r approx. 99000 were observed. By crossed immunoelectrophoresis of the polyacrylamide gel, both bands reacted with anti-plasminogen serum. After reduction, two broad and



EXPLANATION OF PLATE 1

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purified plasmin–inhibitor complex before and after reduction, and identification of the protein bands by immunoelectrophoresis into agarose gel

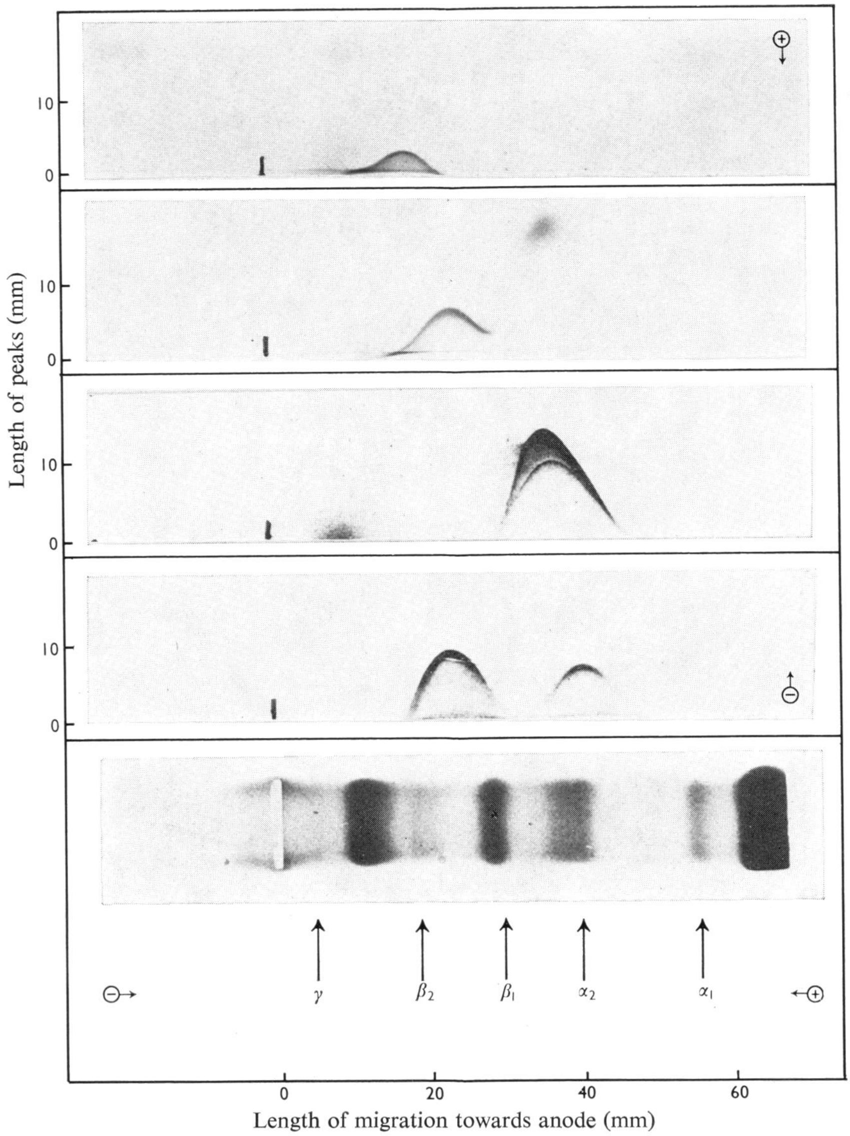
The apparent relative molecular masses of the different bands were determined by reference to the migration of marker proteins and Bromophenol Blue. Primary electrophoresis was as follows: about 12μg of the complex was applied in 40μl of sodium phosphate buffer (0.1 mol/l), pH 7.0; a constant current of 14mA per tube (cross-sectional area 12.6mm^2) was applied for 3h. The second-dimension electrophoresis was in Tris/barbiturate buffer, pH 8.6; a potential gradient of 1V/mm was applied for 17h. For further details, see under 'Methods'.



EXPLANATION OF PLATE 2

Antibodies of an antiserum against a plasmin–inhibitor complex, plasminogen and an α_2 protein, demonstrated by crossed immunoelectrophoresis

(1) Isolated plasmin–inhibitor complex, (2) normal plasma, (3) purified native plasminogen and (4) urokinase-activated plasma. The samples (5μl) were subjected to crossed immunoelectrophoresis. Both dimensions were with a potential gradient of 1V/mm, the first for 2h the second for 17h. The second-dimension electrophoresis was run into agarose gel with anti-complex serum at a volume fraction of 0.04. Antibodies against the plasmin–inhibitor complex with post-β_1 mobility are shown in (1) and (4), against plasminogen (with β_2 mobility) in (2) and (3) and against the inhibitor (with α_2 mobility) in (2) and (4). The protein fractions of the first-dimension electrophoresis of normal plasma are shown in (5). Urokinase-activated plasma was prepared by addition of 0.15vol. of urokinase to plasma to a final concentration of urokinase of 104×10^3 Ploug units/l and keeping it at 37°C for 30min.



EXPLANATION OF PLATE 3

Transformation in plasma by urokinase of plasminogen into a plasmin–inhibitor complex, demonstrated by crossed immuno-electrophoresis

(1) and (3) Normal plasma; (2) and (4) urokinase-activated plasma. The samples (5μl) were subjected to crossed immuno-electrophoresis. Both dimensions were with a potential gradient of 1 V/mm, the first for 2h the second for 17h. The second-dimension electrophoresis was run into agarose gel with anti-plasminogen serum (1,2) and with plasminogen-adsorbed anti-complex serum (3, 4) at volume fractions of 0.025 and 0.04 respectively. The protein fractions of the first-dimension electrophoresis of normal plasma is shown in (5). Whereas plasminogen has β_2 mobility (1) and the inhibitor α_2 mobility (3), the complex has post-β_1 mobility and reacts with both antibodies (2, 4). The half-bow precipitate with α_2 mobility (2) represents a plasmin–α_2 macroglobulin complex (cf. Müllertz, 1974).

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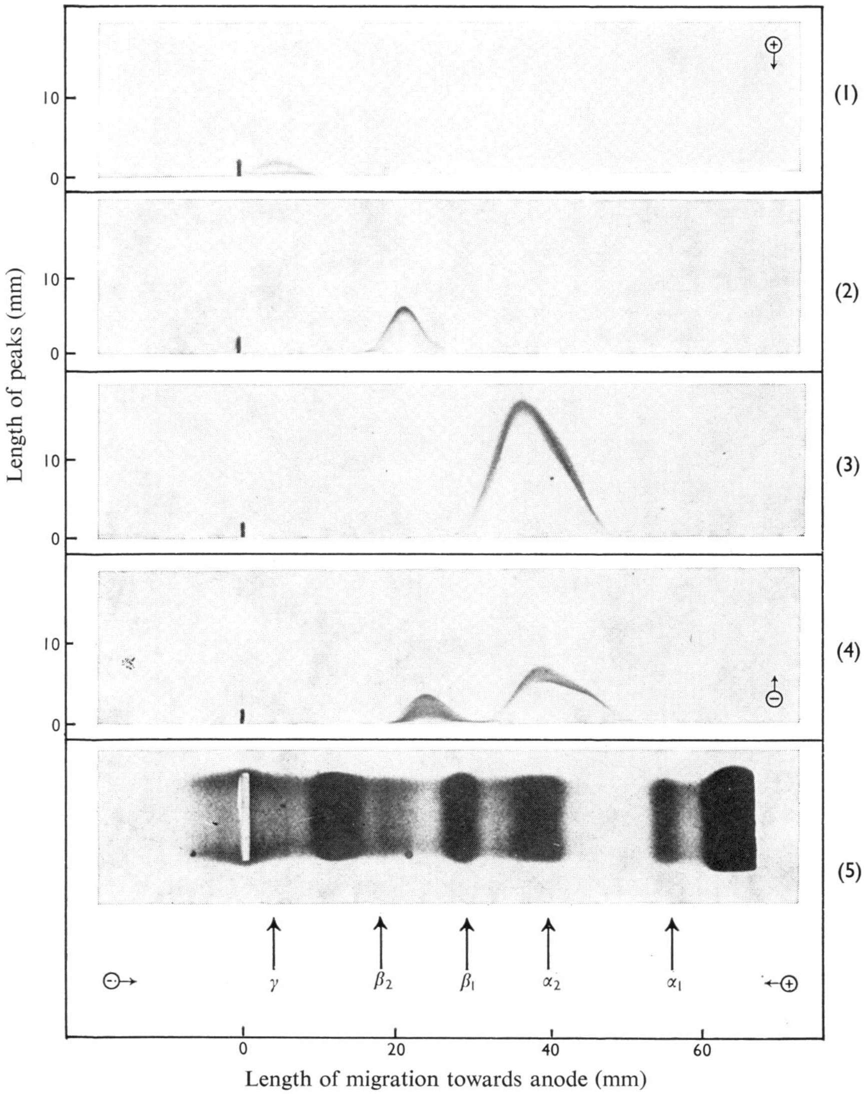

EXPLANATION OF PLATE 4

Formation of a complex between purified plasmin and the primary plasmin inhibitor, demonstrated by crossed immunoelectrophoresis

The systems are a partially purified preparation of the inhibitor (3), purified plasmin (1) and a mixture of purified plasmin (final concentration 0.3 μmol/l) and the inhibitor preparation (final volume fraction 0.10), with excess of inhibitor and zero enzymic activity (2, 4). The samples were concentrated about three times by ultrafiltration and subjected to crossed immunoelectrophoresis. Both dimensions were with a potential gradient of 1 V/mm, the first for 2 h the second for 17 h. The second-dimension electrophoresis was run into agarose gel with anti-plasminogen serum (1, 2), and with plasminogen-adsorbed anti-complex serum (3, 4). The protein fractions of the first-dimension electrophoresis of normal plasma are shown in (5). The formation of a complex with post-β_1 mobility by plasmin (with γ mobility) and by the inhibitor in excess (with α_2 mobility) is demonstrated.

partly confluent bands with M_r approx. 72000 and 66000 and two relatively faint bands with M_r about 57000 and 9000 were observed. By crossed immunoelectrophoresis of the gel a rather broad precipitate peak with anti-plasminogen serum was observed corresponding to the region of the band with M_r approx. 66000. No reaction was detectable with plasminogen-adsorbed anti-complex serum. Controls consisting of crossed immunoelectrophoresis in agarose gel showed that sodium dodecyl sulphate destroyed the antigenicity of the inhibitor component but not the plasmin component of the complex.

Antibodies of a rabbit immune serum against the plasmin–inhibitor complex

Two immune sera were examined for antibodies against proteins of human plasma. Fresh human plasma was subjected to crossed immunoelectrophoresis with the immune serum present in the second-dimension gel. Two distinct antigen–antibody peak precipitates were apparent with β_2 and α_2 mobilities (Plate 2, panel 2). The peak with β_2 mobility was identified as a plasminogen antigen–antibody precipitate for the following reasons. A peak with the same mobility was formed by crossed immunoelectrophoresis of purified native plasminogen against the anti-complex serum (Plate 2, panel 3); after addition of the plasminogen activator, urokinase, the peak with β_2 mobility was converted into a peak with post-β_1 mobility (Plate 2, panel 4). Further, after adsorption of the immune serum to purified plasminogen, the β_2 peak of normal plasma disappeared and only the α_2 peak remained (Plate 3, panel 3). Obviously, the anti-complex serum predominantly contained antibodies against plasminogen+plasmin and a single α_2 protein. One of the two sera had a rather low titre of antibodies against plasminogen.

Formation of a plasmin–inhibitor complex in normal plasma by urokinase

Urokinase was added to normal human plasma at conditions known to convert plasminogen in plasma into a plasmin–inhibitor complex with post-β_1 mobility and into a plasmin–α_2 macroglobulin complex and to initiate degradation of fibrinogen in about 30min at 37°C (Müllertz, 1974). Both normal plasma and urokinase-activated plasma were subjected to crossed immunoelectrophoresis. With anti-plasminogen serum in the second-dimension gel the conversion of plasminogen into a plasmin–inhibitor complex with post-β_1 mobility and into a plasmin–α_2 macroglobulin complex with α_2 mobility was demonstrated (Plate 3, panel 1 and 2). With plasminogen-adsorbed anti-complex serum in the gel, normal plasma produced a single peak with α_2 mobility (Plate 3, panel 3). Addition of urokinase resulted in the formation of a new peak with post-β_1 mobility, and the area of the α_2 peak was decreased to about 0.3 (Plate 3, panel 4). The same changes were apparent with unadsorbed anti-complex serum (Plate 2, panels 2 and 4). The peaks with α_2 and post-β_1 mobility observed, after activation by urokinase, with anti-complex serum were connected by a continuous precipitation line indicating immunological identity (Plate 2, panel 4). The results strongly indicate that plasminogen was converted into plasmin, which combined with an α_2 protein forming a complex with β_1 mobility, decreasing the concentration of unbound antigenic α_2 protein to about 0.3 of the original value.

Elution characteristics by gel filtration of different proteinase inhibitors in plasma

Normal human plasma was subjected to analytical Sephadex G-200 gel filtration. The elution pattern of protein and inhibitors is shown in Fig. 1. The protein reacting with the plasminogen-adsorbed anti-complex antibodies was eluted, with a maximum at a K_D of approx. 0.35, distinctly different from all known proteinase inhibitors except anti-chymotrypsin. This inhibitor, however, is known to be highly

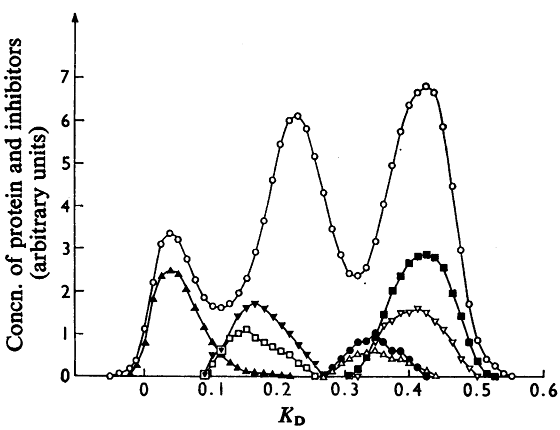

Fig. 1. *Gel-filtration distribution of the known proteinase inhibitors and of the primary plasmin inhibitor in normal human plasma*

Analytical gel filtration was carried out on a column (1.0m×490mm²) of Sephadex G-200 at 3°C. The eluent was Tris/HCl, pH7.6, and the flow rate 11ml/h. Normal human plasma (3ml) was applied. The concentration of protein in the fractions (○) was determined by photometry at 280nm. The concentration of the inhibitors in the fractions was determined by electroimmunoassay in agarose gel with specific immunoglobulins against α_2 macroglobulin (▲), inter-α-inhibitor (□), C_1-esterase inhibitor (▼), primary plasmin-inhibitor (●), anti-chymotrypsin (△), anti-thrombin III (▽) and α_1 anti-trypsin (■). Plasminogen-adsorbed anti-complex serum was used to assay the primary plasmin inhibitor.

specific for chymotrypsin (Heimburger & Haupt, 1965), and the plasmin–α_2 inhibitor complex did not react with anti-(anti-chymotrypsin) serum. An approximate value for M_r of the α_2 inhibitor was calculated from the K_D values to be 75000 (mean); mean ± S.D. was 69000–82000.

Properties of the primary inhibitor of plasmin

Rate of reaction with plasmin. An estimate of the rate of the reaction between plasmin and inhibitor was obtained by mixing inhibitor and plasmin and determining the residual enzymic activity as a function of time. Plasmin and inhibitor preparation were kept separately at 30°C, mixed, and assayed immediately after mixing and after 3, 6 and 60min. The concentrations of plasmin and inhibitor in the mixture were 0.3 μmol/l and 0.15 μmol/l respectively, in Tris/lysine buffer, pH 7.6, the residual enzymic activity was approx. 0.5 of the plasmin control. The experiment showed that the inhibition was complete immediately after mixing and that it did not progress further for 60min at 30°C. The activity of the plasmin control remained constant for the same time.

Titration of plasmin with the inhibitor. The concentration of functionally active plasmin inhibitor in the preparation was determined by titrating a solution of purified activator-free plasmin with the inhibitor preparation, in principle as described by Green (1953) and Ganrot (1967*a*). The original concentration of plasmin was determined by active-site titration. A graph of the residual enzymic activity (catalytic enzyme concentration) versus the volume fraction of the inhibitor preparation in the final mixture was linear until zero plasmin activity was reached, and indicated a strong binding of inhibitor to plasmin (Fig. 2). The concentration of the inhibitor in the material was calculated from the final concentration of plasmin divided by the final volume fraction of the inhibitor material at zero activity, i.e. the intercept on the abscissa. The intercept was calculated by regression analysis. The concentration of functionally active inhibitor [c(f)I] was estimated to be 3.90 μmol/l (S.D. 0.11 μmol/l; $n = 10$). Similar results were obtained with the crude preparation of the inhibitor. In this case c(f)I was 2.53 μmol/l (S.D. 0.06 μmol/l; $n = 10$).

A mixture of purified plasmin and the partially purified inhibitor preparation with a total inhibition of enzymic activity was analysed by crossed immunoelectrophoresis. With anti-plasminogen serum in the second-dimension gel the formation of a plasmin–inhibitor complex with post-β_1 mobility was apparent. With plasminogen-adsorbed anti-α complex serum in the gel, the participation of the antigenic α_2 protein in the formation of the complex was demonstrated (Plate 4).

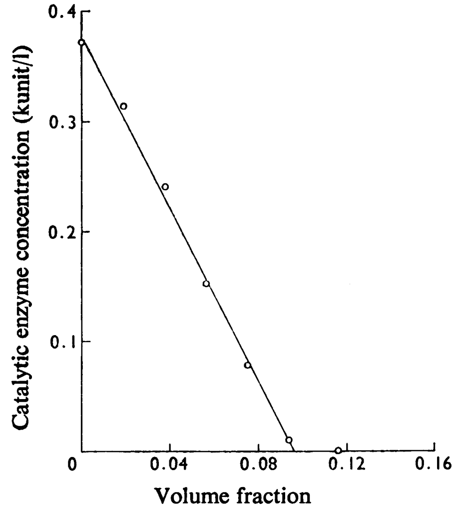

Fig. 2. *Titration of purified plasmin with the primary plasmin inhibitor*

Mixtures of purified plasmin and the partially purified inhibitor preparation in Tris/lysine buffer, pH 7.6, were incubated for 2min at 30°C, and added to the substrate benzoylarginine ethyl ester. The final concentrations were: plasmin, 0.375 μmol/l; benzoylarginine ethyl ester, 0.67 mmol/l; lysine, 0.33 mol/l; Tris/HCl, 0.15 mol/l, pH 7.6. The ordinate is the residual catalytic enzyme concentration of the mixtures (kunit/l), and the abscissa the volume fraction of the inhibitor preparation in the final mixture.

Content of antigenic but inactive inhibitor in the preparations

The concentration of antigenic inhibitor protein [c(ag)I] in both preparations was also estimated by electroimmunoassay with plasminogen-adsorbed anti-complex serum in the gel, and expressed in arbitrary units. The ratio c(f)I/c(ag)I was 0.65 in the partially purified preparation, when the ratio was defined as 1.0 in the crude preparation. The results indicated a partial denaturation of the inhibitor by the purification procedures.

Quantitative crossed immunoelectrophoresis was carried out (Weeke, 1973) of the crude preparation alone and of mixtures of purified plasmin (in excess and in equivalent amounts) and the preparation, demonstrated that about 0.4 of the antigenic and inhibitor protein in this preparation appeared to be functionally inactive.

Other inhibitors in the crude preparation

The crude preparation contained relatively small amounts of the other inhibitors in plasma. Among these anti-chymotrypsin and inter-α-inhibitor do not inhibit plasmin (Heimburger *et al.*, 1971), and anti-thrombin III (Highsmith & Rosenberg, 1974; Crawford & Ogston, 1975) and α_1 anti-trypsin (Rimon *et al.*, 1966; Hercz, 1974) are slow inhibitors of plasmin. Further, the esterase activity of plasmin is only slightly inhibited by α_2 macroglobulin (Ganrot, 1967*b*). The inhibition of plasmin by α_2 macroglobulin (Ganrot, 1967*b*; Müllertz, 1972, 1974), anti-thrombin III (α_2 protein) (Highsmith & Rosenberg, 1974) α_1 anti-trypsin (Hercz, 1974) and C_1-esterase inhibitor (α_2 protein) (Harpel & Cooper, 1975) is associated with the formation of undissociable equimolar complexes. The non-involvement of these inhibitors in the titration of plasmin was secured by crossed immunoelectrophoresis of a reaction mixture with no enzymic activity and a small excess of inhibitor in agarose gel with specific antibodies against these inhibitors. Small single homogeneous precipitate peaks with electrophoretic mobilities characteristic of the various inhibitors but different from that of the complex between plasmin and the primary plasmin inhibitor were observed. Apart from this, the pattern was similar to that of Plate 4.

Concentration of the plasmin inhibitor in human plasma

The ratio of the concentration of the inhibitor antigen in samples of normal plasma and in the crude inhibitor preparation was estimated by quantitative electroimmunoassay. On the pre-supposition that the ratio of antigen to functionally active inhibitor was the same in the two materials, the concentration of the inhibitor in plasma was calculated from:

$$\frac{[c(\text{ag})\text{I, plasma}]}{[c(\text{ag})\text{I, prep.}]} \times [c(\text{f})\text{I, prep.}]$$

The concentration of the primary plasmin inhibitor in plasma was estimated to be 1.13μmol/l [S.D. 0.05μmol/l: n = number of plasma samples from healthy subjects = 9 (four men and five women)].

Antibodies of the immune sera prepared by D. Collen and by us

The adsorbed immunoglobulin solution given by D. Collen and the adsorbed anti-complex antiserum prepared by us were tested by an Ouchterlony gel-immunodiffusion test. Normal human plasma (10μl) was applied to the central hole and the two antibody solutions alternately in four peripheral holes (10μl each). Both solutions produced single distinct and merging precipitates, demonstrating immunological identity. Both solutions also reacted by the same test with the partially purified preparation of the primary plasmin inhibitor.

Discussion

Human plasma contains at least six different proteins that are inhibitors of serine proteinases. Among these, anti-chymotrypsin is highly specific for chymotrypsin (Heimburger & Haupt, 1965), whereas the other inhibitors have more polyvalent affinities. In their purified states these inhibitors, except inter-α-inhibitor, inhibit plasmin (Heimburger *et al.*, 1971).

Whereas α_2 macroglobulin reacts rapidly with plasmin (Ganrot, 1967*b*), anti-thrombin III (Highsmith & Rosenberg, 1974) and α_1 anti-trypsin (Rimon *et al.*, 1966; Hercz, 1974) are slow ('progressive') inhibitors of plasmin. The rate of reaction of C_1-esterase inhibitor with plasmin is not known, but the inhibitor is a slow inhibitor of the serine plasma proteinase kallikrein (Fritz *et al.*, 1972).

Reports have appeared on other proteinase inhibitors in human plasma. Aoki & von Kaulla (1971) and Hedner (1973) reported that an inhibitor of the activation of plasminogen was present in a protein fraction eluted with the early 4S-fraction proteins by gel filtration. The preparations containing the Aoki & von Kaulla (1971) had some anti-plasmin activity, whereas that containing the Hedner (1973) inhibitor had none. Gallimore (1975) found that some fractions, obtained by chromatography of human plasma, inhibited plasmin strongly in a clot-lysis assay, and that these fractions did not contain any of the known inhibitors. The inhibitor was claimed to have inter-α-electrophoretic mobility.

In plasma, the relative importance of the plasmin inhibitors must depend on their concentrations, relative affinities towards, and rates of reaction with, plasmin. The dominating role of α_2 macroglobulin and the non-involvement of α_1 anti-trypsin in blood *in vivo* was demonstrated by Niléhn & Ganrot (1967).

Studies on human post-mortem plasma (Müllertz, 1972) and on urokinase-activated human plasma (Müllertz, 1974) have demonstrated the well-known formation of a complex between plasmin and α_2 macroglobulin, and, in addition, the formation of an enzymically inactive and undissociable complex between plasmin and a hitherto unknown inhibitor, apparently with a higher avidity than α_2 macroglobulin for plasmin. No complexes between plasmin and α_1 anti-trypsin, anti-thrombin III, inter-α-inhibitor and C_1-esterase inhibitor were detectable. In a preliminary communication Collen (1974) reported on a plasmin–α_1 anti-plasmin complex which was different from the well-known plasmin–α_2 macroglobulin complex. Probably the anti-plasmin of the former complex is the same as the protein with α_2 mobility described by Collen *et al.* (1975). In that paper, crossed immunoelectrophoresis was used to demonstrate the presence in plasma of a protein with α_2 mobility that complexed with plasmin, when generated by urokinase in plasma, in principle in a way similar to that described by Müllertz (1974) and in the present paper. Experiments on a sample of immunoglobulin kindly provided by Dr. Collen and on the antiserum described here indicate that both antibody solutions reacted with the same protein in human plasma.

In the present study the plasmin–inhibitor complex was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. If it was not reduced, the M_r of the plasmin–inhibitor complex was approx. 130000. The other component in the material with M_r approx. 99000 was probably a degraded plasmin–α_2 inhibitor complex. The M_r of plasmin has been reported to be 75000 (Robbins *et al.*, 1973), 81000 (Sjöholm *et al.*, 1973; Walther *et al.*, 1974) and 85000 (Wiman & Wallén, 1975). Consequently, the M_r of the plasmin-bound inhibitor should be 45000–55000. It should be pointed out, however, that estimates of M_r by this procedure without reduction are unreliable.

On reduction, plasmin is dissociated into two chains (Robbins *et al.*, 1967). On sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the M_r of the heavy chain was found to be 63000 and that of the light chain 25000 (Wiman & Wallén, 1973). The active site is located in the plasmin/light chain (Summaria *et al.*, 1967). Therefore the two main bands observed after reduction of the material with the complex may represent an acyl-enzyme complex between plasmin/light chain and inhibitor/heavy chain (M_r approx. 72000) and a plasmin/heavy chain (M_r approx. 66000) reacting with anti-plasminogen serum only. In that case the M_r of the inhibitor component in the reduced complex should be approx. 47000. The component with M_r approx. 9000 may be an inhibitor–light chain and the component with M_r approx. 57000 may be a complex between plasmin–light chain and a degraded inhibitor.

The reaction between plasmin and the inhibitor was fast, probably less than 15 s at concentrations of about 0.3 and 0.15 μmol/l respectively and 30°C. The reaction did not progress further if the mixture was left for 1 h. The complex was inactive and could not be dissociated by gel filtration and gel electrophoresis (Müllertz, 1972, 1974). Therefore the following working hypothesis for the reaction between plasmin and the inhibitor may be proposed. Plasmin splits a peptide bond in the inhibitor and an ester bond is formed between the serine residue of the active site of plasmin and the *C*-terminus of the heavy chain of the inhibitor. The light chain of the inhibitor remains attached to the complex by a disulphide bridge. Through reduction, the disulphide bonds are broken and the complex is dissociated into plasmin–heavy chain, inhibitor–light chain and a complex of a plasmin–light chain and an inhibitor–heavy chain. A similar mechanism was suggested by D. Collen (personal communication). The mechanism of reaction is analogous to the general three-step mechanism of enzymic reaction of the serine proteinases (Bender & Kezdy, 1965), also demonstrated to be true for plasmin (Christensen & Müllertz, 1974).

Such a general mechanism for the reaction of inhibitors with serine proteinases has been proposed by Laskowski & Sealock (1971). The mechanism has not been demonstrated to apply to any of the known proteinase inhibitors in plasma. The discrepancy between the apparent M_r of the inhibitor found by gel filtration (approx. 75000) and that calculated from the M_r of the complex (approx. 45000–50000) and the probable presence of a partially degraded plasmin–inhibitor complex in the preparations may indicate a more complex mechanism of reaction between plasmin and the inhibitor, possibly encompassing a variable proteolytic degradation of the inhibitor.

Both preparations of the inhibitor contained antigenic material, which was not bound by an excess of plasmin. Probably the inhibitor in normal plasma is also heterogeneous, consisting of functionally active and inactive material. A complete activation of the excess of plasminogen present in normal plasma converted only about 0.7 of the antigenic material into a complex with plasmin. The remaining antigenic material appeared to represent the most anodic part of the total material in normal plasma (Plates 2 and 3).

The concentration of the α_2 inhibitor in normal human plasma was estimated to be about 1.1 μmol/l. The concentration of plasminogen in plasma is about 2.5 μmol/l (Rabiner *et al.*, 1969). In a previous study (Müllertz, 1974) it was clearly demonstrated that addition of urokinase to plasma induced primary formation of a complex between plasmin and the α_2 inhibitor and subsequent formation of a complex between plasmin and α_2 macroglobulin. With small amounts of urokinase only the primary complex was formed, the concentration of activatable plasminogen in plasma was decreased to between 0.5 and 0.7 of the original value, and no lysis of fibrinogen occurred. Five times as much urokinase, or the presence of fibrin, enhanced the reaction so that the complex between plasmin and α_2 macroglobulin was also formed. These studies suggest that the α_2 inhibitor is the primary plasmin inhibitor, which is capable of binding rapidly and irreversibly about half of the amount of plasmin that can be generated in the plasma. Only when all the primary inhibitor is saturated, is a complex between plasmin and α_2 macroglobulin formed and fibrinolysis and fibrinogenolysis can occur.

This work was supported by a grant from Statens Laegevidenskabelige Forskningsråd Jh. no. 512-3683. The valuable assistance of Berit Madsen is appreciated.

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