Partial Purification and Characterization of a New Fast-Acting Plasmin Inhibitor from Human Platelets

EVIDENCE FOR NON-IDENTITY WITH THE KNOWN PLASMA PROTEINASE INHIBITORS

Mogens SANDBJERG HANSEN and Inge CLEMMENSEN

Department of Clinical Chemistry, University of Copenhagen, Hvidovre Hospital,
DK-2650 Hvidovre, Denmark

(Received 31 August 1979)

An inhibitor of the plasma proteinase plasmin (EC 3.4.21.7) was partially purified from washed and lysed human blood platelets by $(\text{NH}_4)_2\text{SO}_4$ fractionation and affinity chromatography on Sepharose-linked purified plasminogen. The material contained none of the known plasma proteinase inhibitors when studied by crossed-immuno-electrophoresis and electroimmunoassay, but inhibited a clot-lysis-time assay and an esterolytic assay that used the synthetic substrate S-2251 (D-Val-Leu-Lys-*p*-nitro-anilide). The inhibitory activity had the same mobility as the α_2 -plasma proteins on preparative agarose-gel electrophoresis. Titration of the inhibitor preparation by active-site-titrated plasmin demonstrated a dissociation constant of approx. 0.1 nM. The inhibition was complete within 1 min. The inhibitor increased the mobility in agarose-gel electrophoresis of purified activator-free plasmin or ^{125}I -labelled plasmin, as demonstrated by crossed-immunoelectrophoresis against specific immunoglobulins against plasminogen or by radioautography. The results strongly suggest the presence in platelets of a plasmin inhibitor different from the known plasma proteinase inhibitors.

Thrombocytes are of major importance for normal haemostasis as well as in unwanted thrombus formation, primarily in the arterial system (Olsson, 1974; Harker & Slichter, 1974). The platelets contribute their plug-forming capabilities, vaso-active amines and a representative selection of plasma proteins to the thrombus formation. But it is now evident that they also serve as important activators and accelerators for the coagulation cascade (Walsh, 1974; Østerud *et al.*, 1977; Miletich *et al.*, 1977). The resultant fibrin deposits are primarily degraded through the action of the serine proteinase plasmin (EC 3.4.21.7).

Plasmin is formed from a plasma precursor, plasminogen, under the influence of specific serine proteinases, plasminogen activators (Astrup, 1966). The plasmin is inactivated by inhibitors present in plasma, primarily the fast acting α_2 -plasmin inhibitor (Moroi & Aoki, 1976; Müllertz & Clemmensen, 1976; Collen, 1976) and α_2 -macroglobulin (Müllertz, 1974), the other plasma proteinase inhibitors appearing to be of little importance under physiological conditions (Müllertz, 1978).

Fibrinolytic inhibitors associated with blood platelets have been described (for a review see

Thorsen *et al.*, 1972). Both inhibition of plasmin and of activator may be the cause of the effect, but this has not been resolved. The tenacious adherence of plasma proteins to platelets and the presence of functional α_2 -macroglobulin and α_1 -antitrypsin in platelet lysates (Nachman & Harpel, 1976; Bagdasarian & Colman, 1978) makes a strict control necessary to ensure that no plasma proteinase inhibitors are present in the investigated preparations.

We describe in the present paper the partial purification and some properties of a fast acting plasmin inhibitor from human blood platelets and demonstrate its non-identity with the known plasma proteinase inhibitors.

Experimental

Materials

Buffers for gel electrophoresis and gel filtration were as follows: Tris, 0.1 M-Tris/HCl/0.05 M-NaCl, pH 7.6 (10.15); Tris/gelatin, 2.5 g of gelatin/litre of Tris; electrophoresis buffer: Tris/barbiturate, 4 mM-Tris/25 mM-diethylbarbituric acid/0.3 mM-calcium

lactate/2 mM- NaN_3 , pH 8.6 (I 0.07). Cell-suspension buffer was modified Tyrodes buffer: 2.7 mM-KCl/137 mM-NaCl/0.4 mM- NaH_2PO_4 /12 mM- NaHCO_3 /5.6 mM-glucose/2 mM- NaN_3 , pH 7.3. Wash buffer was Tyrodes buffer containing 1 mM-trisodium citrate.

Human blood was drawn from normal donors or patients with myeloproliferative diseases into 5.2 mM-citric acid/11.2 mM-sodium citrate/20.4 mM-glucose. The results described were the same irrespective of the source of blood.

Agarose was obtained from L'Industrie Biologique Française, S.A., Genevilliers, Seine, France. Sephadex, Blue Dextran (M_r 2×10^6) and chromatographic equipment were obtained from Pharmacia, Uppsala, Sweden.

Protein standard plasma with known concentrations of the relevant plasma proteins was from Behringwerke, Marburg/Lahn, W. Germany. Mono-specific rabbit immunoglobulins against human plasma proteins were purchased from Dako Immunoglobulins, Copenhagen, Denmark. Mono-specific rabbit immunoglobulins against the α_2 -plasmin inhibitor was produced and controlled as described by Müllertz & Clemmensen (1976). Fibrinogen for fibrin-clot-lysis assays was bovine plasminogen-rich fibrinogen kindly supplied by Dr. J. Ploug, Leo Pharmaceuticals, Copenhagen, Denmark. Urokinase and bovine thrombin were also from Leo Pharmaceuticals.

The bovine proteinase inhibitor aprotinin (Trasylol) was from Bayer A.G., Leverkusen, W. Germany. 6-Aminohexanoic acid was from Fluka A.G., Buchs, Switzerland. The synthetic plasmin substrate S-2251 (D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride) was a gift from Kabi-Vitrum, Copenhagen, Denmark. *p*-Nitrophenyl-*p'*-guanidinobenzoate was from ICN Pharmaceuticals, Cleveland, OH, U.S.A.

Methods

Immunochemical techniques. The concentration of plasma proteinase inhibitors, plasma proteins and plasminogen were determined by quantitative electroimmunoassay in agarose gel containing specific antibodies as described by Laurell (1972). Crossed-immunoelectrophoresis was performed in agarose gel, essentially as described by Ganrot (1972). Double immunodiffusion was performed by the method of Ouchterlony (1953).

Ultrafiltration. This was done in a stirred cell (Amicon, Lexington, MA, U.S.A.) with a PM10 membrane, which retained molecules with M_r higher than 10000.

Radiolabelling of proteins. This was performed essentially as described by Thorell & Johansson (1971) by using immobilized lactoperoxidase (Gilbert & Wachsmann, 1976). Less than 0.5 g-atom of iodine was incorporated per mol of protein.

Determination of apparent M_r by gel chromatography. This was performed as described by Andrews (1965). The column was calibrated as described by Müllertz & Clemmensen (1976). Pooled and concentrated fractions from the affinity-chromatography procedure (19 mg of protein in 2 ml) or the $(\text{NH}_4)_2\text{SO}_4$ -precipitated preparation were applied to a Sephadex G-100 column ($0.62 \text{ m} \times 200 \text{ mm}^2$), and 2 ml fractions were collected. Each fraction was assayed for inhibitor in a clot-lysis-time assay. Protein fractions were indicated by their partition coefficient (K_{av}).

Determination of apparent relative molecular mass (M_r) by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The technique was essentially as described by Weber & Osborn (1975) and Müllertz & Clemmensen (1976).

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

Determination of the concentration of plasminogen and proteinase inhibitors in plasma. The mass concentrations of the known plasma proteinase inhibitors and plasminogen in the preparation were determined by electroimmunoassay by reference to a protein standard plasma. The substance concentrations were calculated by means of the relative molecular masses: α_2 -macroglobulin (M_r 725 000), component C1-inactivator (M_r 104 000), α_1 -antichymotrypsin (M_r 69 000), antithrombin III (M_r 62 000), α_1 -antitrypsin (M_r 61 000), the primary α_2 -plasmin inhibitor (M_r 70 000) and plasminogen (M_r 90 000).

Analytical agarose-gel electrophoresis. This was performed as described by Johansson (1972).

Preparative agarose-gel electrophoresis. The procedure was essentially as described by Müllertz (1974). After extraction of the proteins from the agarose gel, they were dialysed against Tris buffer and concentrated to a protein content of approx. 0.5 g/litre.

Preparation of plasminogen. Plasminogen was prepared from human plasma by affinity chromatography on lysine-Sepharose (Deutsch & Mertz, 1970) as described by Müllertz & Clemmensen (1976).

Preparation of plasmin. Plasmin was prepared in an activator-free state as described by Wiman & Wallén (1973) and Müllertz & Clemmensen (1976). In some experiments plasmin was prepared by activation of plasminogen by traces of urokinase as described by Müllertz (1974), by using a molar ratio of plasminogen/urokinase of approx. 2000:1 at a plasminogen concentration in water of approx. $50 \mu\text{M}$. For the titration experiments plasmin was produced by incubating $50 \mu\text{l}$ of plasminogen (70 – $85 \mu\text{M}$) and $50 \mu\text{l}$ of 6-aminohexanoic acid (150 mM) with $75 \mu\text{l}$ of urokinase (2×10^6 Ploug units;

4.2×10^6 Ploug units are approx. $1 \mu\text{mol}$ of urokinase). The mixture was incubated for 150 s at 37°C and diluted with $525 \mu\text{l}$ of 6-aminohexanoic acid (50 mM) at 0°C . This stock solution was further diluted with Tris/gelatin buffer at 0°C .

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

Crude preparation of the thrombocyte plasmin inhibitor. Blood stabilized with citric acid/sodium citrate/glucose (usually 3–4 litres at a time) was centrifuged for 5 min at 20°C at $800 g_{\text{av}}$ in a Beckman J-6 centrifuge equipped with a JS-4.2 rotor. The supernatant was carefully collected, and the platelets therein were washed by repeated sedimentation and resuspension in wash buffer essentially as previously described (Bang *et al.*, 1975). The centrifuge used in the washing procedures and the following steps was a Sorvall RC5 equipped with an SS-34 rotor. The platelets were resuspended five times in fresh buffer at 20°C . The final platelet suspension, in modified Tyrodes buffer, containing approx. 30% of the original amount of platelets, was frozen and thawed four times and centrifuged at 4°C for 60 min at $10000 g_{\text{av}}$. Solid $(\text{NH}_4)_2\text{SO}_4$ was added slowly to the supernatant, with stirring, at 4°C to a final concentration of 1.25 M. After 1 h the precipitate was separated by centrifugation at 4°C for 15 min at $10000 g_{\text{av}}$. $(\text{NH}_4)_2\text{SO}_4$ was added to this supernatant to a final concentration of 2.73 M. The precipitate was isolated as above and dissolved in Tris buffer. This solution was extensively dialysed against Tris buffer and concentrated by ultrafiltration.

Determination of the concentration of the inhibitor. Method 1. The inhibition of fibrinolysis induced by plasmin or activator was determined by a fibrin-clot-lysis-time assay as described by Thorsen (1973), but with Tris buffer instead of sodium barbital buffer. A fibrinogen solution (0.3 ml , $18.4 \mu\text{M}$) was mixed with 0.1 ml of inhibitor preparation or Tris buffer and clotted with a mixture of 0.05 ml of thrombin solution (5×10^5 NIH units/litre) and 0.05 ml of urokinase solution (3.3×10^4 – 5×10^5 Ploug units/litre). (One NIH unit is the amount of thrombin that coagulates a standardized human fibrinogen solution in 45 s at 25°C .)

Method 2. The concentration of the inhibitor was determined from the amount of active-site-titrated plasmin inactivated in mixtures of inhibitor and plasmin in excess. The residual amount of plasmin was estimated by use of the synthetic tripeptide S-2251, which has a high specificity for plasmin (Mattler & Bang, 1977) with a K_m of 0.12–0.25 mM (Friberger *et al.*, 1977; Christensen *et al.*, 1979). The final concentrations in the reaction

mixture were plasmin 32 nM, 6-aminohexanoic acid 48 mM and compound S-2251 $360 \mu\text{M}$. The change in A_{410} was recorded in a Beckman spectrophotometer model 25 with microcuvettes and a Beckman recorder.

Partial purification of the thrombocyte plasmin inhibitor. This was done by $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by affinity chromatography on Sepharose-linked purified plasminogen. The activator-free purified plasminogen ($4 \mu\text{mol}$) was coupled to CNBr-activated Sepharose 4B (30 g) (Axén *et al.*, 1967). The amount of plasminogen was estimated from the A_{280} , by using a specific absorbance coefficient of $138510 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. The column ($0.23 \text{ m} \times 530 \text{ mm}^2$) was equilibrated at 4°C with Tris buffer. The non-dialysable protein material (107 mg in 15 ml) was applied. Unadsorbed protein was eluted by percolating 250 ml of Tris buffer through the column (70 ml/h). The adsorbed proteins were eluted with the same buffer containing 6-aminohexanoic acid (5 mM, 150 ml).

Production of a complex between plasmin and the thrombocyte plasmin inhibitor. The partially purified inhibitor preparation was incubated with activator-free plasmin for 150 s at 20°C . Residual plasmin activity was then inhibited by the addition of the bovine proteinase inhibitor aprotinin. This preparation as well as a preparation of plasmin incubated with buffer was subjected to crossed-immunoelectrophoresis with immunoglobulins against plasminogen in the second-dimension gel. The final concentrations in the incubation mixture were plasmin 230 nM and inhibitor about 250 nM. Urokinase and ^{125}I -labelled plasminogen were also incubated with the inhibitor preparation and with Tris/gelatin buffer. This mixture was subjected to agarose-gel electrophoresis. The gels were dried and radioautographed on X-ray film. The final concentrations in the incubation mixture were plasminogen 230 nM, urokinase 90×10^3 Ploug units/litre and inhibitor about 250 nM.

The rate of complex-formation was also studied by using the same concentrations in the reaction mixture. Samples were withdrawn at fixed time intervals and subjected to crossed-immunoelectrophoresis. Residual plasmin activity was inhibited as above. Prolonged incubation or the use of plasmin in excess caused degradation of the complex.

Results

Protein characterization of the crude inhibitor preparation

The material with anti-fibrinolytic activity from the $(\text{NH}_4)_2\text{SO}_4$ precipitation, containing approx. 25–200 nM-inhibitor in several different preparations, was studied by crossed-immunoelectrophoresis and by electroimmunoassay in agarose gel

containing antibodies against one of the following proteins: plasminogen, α_1 -antitrypsin, antithrombin III, the primary α_2 -plasmin inhibitor, α_1 -antichymotrypsin, inter- α -trypsin inhibitor, component C1 inactivator and α_2 -macroglobulin. No precipitates were detectable in the gels containing immunoglobulins against the inhibitors or plasminogen. This was done to ensure that the inhibitory activity represented a blood platelet protein and not a contaminating plasmin inhibitor from plasma. The sensitivity of the assay for the known plasma plasmin inhibitors were: α_2 -plasmin inhibitor, $0.01\mu\text{M}$; α_2 -macroglobulin, $0.05\mu\text{M}$; α_1 -antitrypsin, $0.04\mu\text{M}$; antithrombin III, $0.04\mu\text{M}$; component C1 inactivator, $0.01\mu\text{M}$. These theoretical maximum concentrations of the plasma plasmin inhibitors cannot account for the plasmin-inhibitory activity in the preparations. No precipitates were formed upon double immunodiffusion against the same immunoglobulins against the same inhibitors. Each batch was checked in this manner. It is not yet known whether the platelet plasmin inhibitor is present in plasma.

Inhibition of the fibrin-clot-lysis time by a crude inhibitor preparation

The $(\text{NH}_4)_2\text{SO}_4$ -precipitated inhibitor preparation caused the lysis time of the fibrin-clot-lysis assay to increase exponentially with a decreasing concentration of urokinase (Fig. 1).

Fig. 1. Clot-lysis-time assay in the presence and absence of the crude inhibitor preparation

Plasminogen-rich fibrinogen solution ($300\mu\text{l}$, $18.4\mu\text{M}$) was mixed with $100\mu\text{l}$ of the crude inhibitor preparation (▲) or Tris buffer (●). The clotting and lysis was induced simultaneously by the addition of thrombin (5×10^5 NIH units/litre) and urokinase (3.3×10^4 – 5×10^5 Ploug units/litre) in $100\mu\text{l}$. A semi-log plot is shown; the broken line indicates that the lysis time at the lowest urokinase concentrations was more than 24 h.

Titration of plasmin by the crude inhibitor preparation

A graph of the residual enzyme activity versus the volume fraction of the $(\text{NH}_4)_2\text{SO}_4$ -precipitated pre-

Fig. 2. Titration of purified plasmin with the crude or partially purified thrombocyte plasmin inhibitor

Mixtures of plasmin and the inhibitor preparation were incubated for 150 s at 20°C and added to the substrate S-2251. The ordinate is the residual enzyme concentration (nM) and the abscissa the volume fraction of the inhibitor preparation in the final mixture.

Fig. 3. Affinity chromatography of the crude inhibitor preparation on Sepharose-linked purified plasminogen

The adsorbed proteins were eluted with 6-amino-hexanoic acid (5 mM) in Tris buffer starting at the arrow. The eluted proteins were pooled between 0 and 75 ml (I), 75 and 250 ml (II), 250 and 340 ml (III) and 240 and 400 ml (IV).

Table 1. *Degree of purification in a representative experiment*

Ten experiments on ten different batches have given comparable results. For details see the text.

	Inhibitor concentration (nM)	Protein concentration (g/l)	Inhibitor (nmol/g of protein)
Platelet lysate	22.5	10.0	2.3
Crude inhibitor preparation	25.4	3.2	7.9
Partially purified inhibitor preparation	15.0	0.7	21.4

paration or the partially purified preparation in the final mixture is shown in Fig. 2. Titration of plasmin by four different inhibitor preparations assuming a 1:1 binding ratio between the inhibitor and plasmin has demonstrated a dissociation constant of approx. 0.1 nM calculated as described by Green & Work (1953) and Bieth (1974).

Preparative agarose-gel electrophoresis of the crude inhibitor preparation

When estimated by a fibrin-clot-lysis-time assay, the inhibitory activity had the same electrophoretic mobility as the α_2 -plasma proteins in three experiments involving three different preparations.

Partial purification of the thrombocyte plasmin inhibitor by affinity chromatography on Sepharose-linked plasminogen

The $(\text{NH}_4)_2\text{SO}_4$ -precipitated inhibitor preparation was subjected to affinity chromatography on Sepharose-linked purified plasminogen. The elution pattern is shown in Fig. 3, and the fractions were pooled as indicated. The pools were dialysed for at least 20 h against Tris buffer and concentrated before further characterization. The protein content and the degree of purification is seen in Table 1. The fibrin-clot-lysis-time assay for the partially purified inhibitor is depicted in Fig. 4, demonstrating inhibition of the activator-induced fibrin-clot lysis by both adsorbed and non-adsorbed proteins. Estimation of the inhibitor concentration by titration with plasmin demonstrated a binding of 21% of the inhibitor to the plasminogen-Sepharose, whereas only 8% of the protein mass was bound. When the pool, which did not bind to the plasminogen-Sepharose, was passed over the regenerated column, less than 4% of the remaining activity was bound.

Demonstration of a complex between plasmin and the $(\text{NH}_4)_2\text{SO}_4$ -precipitated and the partially purified inhibitor preparation

The conversion of plasmin into a broad plasmin-inhibitor complex with the same electrophoretic mobility in agarose as the β_2 -plasma proteins was demonstrated by crossed-immunoelectrophoresis (Fig. 5). This was also demonstrated with radio-

Fig. 4. Clot-lysis-time assay of the inhibitor-containing pools II and IV from Fig. 3

The pools were dialysed against Tris buffer and concentrated to the same volume as the starting material. The assay was performed as described in Fig. 1 with starting material (▲), pool II (non-adsorbed proteins) (■), pool IV (adsorbed proteins) (●) and Tris buffer (○). The concentration of the inhibitor in pool II is approx. 3 times that in pool IV. A semi-log plot is shown; the broken line indicates that the lysis time at the lowest urokinase concentrations was more than 24 h.

labelled plasmin (Fig. 6). The conformation and localization of the complex was the same irrespective of the inhibitor preparation used. A complex between plasmin and the fast acting α_2 -plasmin inhibitor and α_2 -macroglobulin, produced as described by Müllertz & Clemmensen (1976), shows a different electrophoretic mobility (Fig. 5).

Rate of enzyme-inhibitor complex-formation and of reaction with plasmin

An estimate of the rate of reaction between plasmin and inhibitor was obtained by two methods. First, the rate of formation of a complex between plasmin and inhibitor was studied in the manner described above by incubating the plasmin and the inhibitor preparation for between 30 s and 30 min.

Fig. 5. Formation of a complex between purified plasmin and the partially purified thrombocyte plasmin inhibitor demonstrated by crossed-immunoelectrophoresis in agarose

The systems are purified plasmin (a) and a complex between purified plasmin and the partially purified inhibitor preparation (b). (c) demonstrates the mobilities of complexes between plasmin and the α_2 -plasmin inhibitor (the cathodic precipitate) and between plasmin and α_2 -macroglobulin (the anodic precipitate). 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 containing anti-plasminogen immunoglobulins. The protein fractions of the first-dimension electrophoresis of normal plasma are shown in (d). The anode is towards the right in the first dimension and towards the top in the second dimension.

The experiment demonstrated that the complex-formation was complete after 30–60 s and that it did not progress further over the next 30 min.

Second, the residual plasmin activity was determined as a function of time after incubation of the plasmin and the inhibitor preparation for between 60 s and 15 min. The residual enzyme activity was approximately half of that of the plasmin control (see under 'Methods'). This experiment demonstrated that the inhibition was complete after 1 min and that it did not progress further over the next 15 min. The activity of the plasmin control remained constant during the same period of time.

Apparent M_r of the inhibitor

An approximate value for M_r was obtained by column chromatography of the $(\text{NH}_4)_2\text{SO}_4$ -precipitated material. The M_r was calculated from the partition coefficients (K_{av}) to be approx. 52 000 (Fig. 7).

Fig. 6. Radioautography of crossed-immunoelectrophoresis prepared as in Fig. 5, of a complex between ^{125}I -labelled plasmin and the partially purified inhibitor preparation

^{125}I -labelled purified plasminogen is seen in (a) and a complex between plasmin and the partially purified inhibitor preparation in (b). Residual plasmin is seen at the application slit in (b).

Fig. 7. Gel chromatography on Sephadex G-100 of the crude inhibitor preparation

The concentration of protein in the fractions was determined by measuring A_{280} (●). The amount of anti-fibrinolytic activity was determined by a clot-lysis-time assay (■).

The material that bound to the plasminogen-Sepharose formed three bands on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under non-reducing conditions. The M_r values of the bands were approx. 125 000, 108 000 and 55 000. After reduction, bands with M_r about 52 000, 39 000 and 34 000 were observed.

Discussion

Seven inhibitors of serine proteinases have been described in human plasma (Müllertz, 1978). α_1 -Antichymotrypsin and inter- α -trypsin inhibitor do not inhibit plasmin (Heimbürger *et al.*, 1971), whereas the remaining inhibitors are all able to bind plasmin in pure systems. Antithrombin III (Highsmith & Rosenberg, 1974), α_1 -antitrypsin (Hercz, 1974) and component C1 inactivator (Harpel &

Cooper, 1975) are slow acting inhibitors of plasmin and appear to be of little importance in whole plasma *in vivo* or *in vitro* (Müllertz, 1978). The primary α_2 -plasmin inhibitor (Moroi & Aoki, 1976; Müllertz & Clemmensen, 1976; Collen, 1976) and α_2 -macroglobulin (Müllertz, 1974; Harpel & Cooper, 1975) are the major inhibitors of plasmin in plasma. Comparatively less is known about the human tissue proteinase inhibitors. The trypsin inhibitor from pancreas (Vogel *et al.*, 1968) and the placental urokinase inhibitor with M_r 48 000 (Holmberg *et al.*, 1978) do not inhibit plasmin.

Blood platelets carry a representative selection of plasma proteins adsorbed to their surface. This is a potential source of error in assessing the intrinsic platelet anti-fibrinolytic activity, and necessitates strict control measures. In this study the presence of the known proteinase inhibitors from plasma in the platelet material was ruled out by immunological methods. Both the crude and the partially purified material were tested by double immunodiffusion and by crossed-immunoelectrophoresis with specific immunoglobulins against the known plasma proteinase inhibitors in the second dimension. The complex between the inhibitor and plasmin was tested in the same manner. Furthermore, the complex had an electrophoretic mobility distinctively different from that of complexes between the primary α_2 -plasmin inhibitor or α_2 -macroglobulin and plasmin. The further characterization of the partially purified platelet plasmin inhibitor demonstrated functional similarities to the fast acting α_2 -plasmin inhibitor in plasma. The rate of reaction with plasmin was fast, probably less than 60 s. The fast acting α_2 -plasmin inhibitor has an equally high reaction rate (Müllertz & Clemmensen, 1976; Christensen & Clemmensen, 1978). This high reaction rate with purified plasmin strongly supports the idea that the inhibitor is primarily a plasmin inhibitor.

The binding characteristics of the inhibitor to plasminogen-Sepharose raises some interesting questions. Only a part, approx. 22%, of the inhibitor activity can be bound to plasminogen-Sepharose, even by repeated passage over the gel. This phenomenon may signify the presence of two inhibitors with different specificities and characteristics. The gel chromatography demonstrates the presence of inhibitory activity of only one molecular mass. Thus it is possible that two slightly different forms of the inhibitor exist, of which only one has affinity for plasminogen, but both are able to inhibit plasmin. The same findings have been described for the fast acting α_2 -plasmin inhibitor in plasma (Clemmensen, 1979a,b). This functional difference cannot be readily explained at this time.

The relative molecular mass of the inhibitor was estimated by gel chromatography to be approx. 50 000. Previously published molecular masses of

platelet anti-fibrinolytic activity are less than 100 000 (Mui *et al.*, 1975), 30 000 (Joist *et al.*, 1976), 35 000 (Ganguly, 1972), 40 000 (Wakabayashi *et al.*, 1970), 45 000 (Murray *et al.*, 1974), 70 000 (Moore *et al.*, 1975) and over 110 000 (McDonagh *et al.*, 1969). Whether the present inhibitor is identical with one or more of these inhibitors is difficult to resolve, since no data are available on the rate of inhibition of plasmin, the complex-formation with plasmin, the possible affinity for plasminogen or the dissociation constant for the inhibitor-plasmin complex. Preliminary experiments might indicate that the complex is dissociable on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, but this has not yet been settled because of the difficulty in obtaining larger amounts of the inhibitor.

From the yield of inhibitor in the preparation an approximate concentration of 1 nmol of inhibitor/ 10^{11} blood platelets (approx. 1.3 ml of packed platelets) could be estimated. This low molar concentration of the inhibitor does not necessarily reflect its importance for the local fibrinolytic activity in a clot. The concentration in the lysates does not necessarily reflect an equally low concentration in the intact platelets, since some platelets are lost during the washing procedures and some undoubtedly have released partially or fully.

The fast acting α_2 -plasmin inhibitor is present in plasma in relatively low concentrations compared with other proteinase inhibitors, but is recognized as being of essential physiological importance for the normal regulation of the fibrinolysis.

The initial phase of thrombus formation involves the growth of a platelet thrombus on subendothelial structures. This platelet thrombus is subsequently reinforced by the formation of fibrin. The presence of a fibrinolytic inhibitor centrally in a forming thrombus will conceivably protect the fibrin from premature degradation. It is thus likely that this inhibitor, which is present in low concentrations, but has a high specificity for, and fast rate of reaction with, plasmin, is an essential modulator of plasmin activity *in vivo*.

This study was supported by a grant from the Arvid Nilsson Foundation. The expert technical assistance of Dorte Vestergaard Winther is gratefully acknowledged. Polycythaemic blood and normal blood platelets were kindly provided by the Department of Haematology, Gentofte University Hospital and the blood bank, Hvidovre University Hospital.

References

- Andrews, P. (1965) *Biochem. J.* **96**, 595–606
- Astrup, T. (1966) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **25**, 42–51

- Axén, R., Porath, J. & Emback, S. (1967) *Nature (London)* **214**, 1302–1304
- Bagdasarian, A. & Colman, R. W. (1978) *Blood* **51**, 139–156
- Bang, N. U., Hansen, M. S. & Heidenreich, R. O. (1975) *Excerpta Med. Int. Congr. Ser.* **357**, 118–126
- Bieth, J. (1974) in *Proc. Int. Res. Conf. Proteinase Inhibitors 2nd* (Fritz, H., Tschesche, H., Greene, L. J. & Truscheit, E., eds.), pp. 463–469, Springer-Verlag, Berlin and New York
- Chase, T. & Shaw, E. (1969) *Biochemistry* **8**, 2212–2234
- Christensen, U. & Clemmensen, I. (1978) *Biochem. J.* **175**, 635–641
- Christensen, U., Sottrup-Jensen, L., Magnusson, S., Petersen, T. E. & Clemmensen, I. (1979) *Biochim. Biophys. Acta* **567**, 472–481
- Clemmensen, I. (1979a) in *Physiological Inhibitors of Coagulation Fibrinolysis* (Collen, D., Wiman, B. & Verstraete, M., eds.), pp. 131–136, Elsevier, Amsterdam
- Clemmensen, I. (1979b) *Thromb. Haemostasis* **42**, 103
- Collen, D. (1976) *Eur. J. Biochem.* **69**, 209–216
- Deutsch, D. G. & Mertz, E. T. (1970) *Science* **170**, 1095–1096
- Friberger, P., Knös, M., Gustavsson, S., Aurell, L. & Claeson, G. (1977) *Thromb. Haemostasis* **38**, 22
- Ganguly, P. (1972) *Clin. Chim. Acta* **39**, 466–468
- Ganrot, P. O. (1972) *Scand. J. Clin. Lab. Invest. Suppl.* **124**, 39–41
- Gilbert, L. R. & Wachsman, J. T. (1976) *Anal. Biochem.* **72**, 480–484
- Green, N. M. & Work, E. (1953) *Biochem. J.* **54**, 347–352
- Harker, L. A. & Slichter, S. J. (1974) *Thromb. Diath. Haemorrh.* **31**, 188–202
- Harpel, P. C. & Cooper, N. R. (1975) *J. Clin. Invest.* **55**, 593–604
- Heimbürger, N., Haupt, H. & Schnick, H. G. (1971) in *Proc. Int. Res. Conf. Proteinase Inhibitors 1st* (Fritz, H. & Tschesche, H., eds.), pp. 1–21, de Gruyter, Berlin
- Hercz, A. (1974) *Eur. J. Biochem.* **49**, 287–292
- Highsmith, R. F. & Rosenberg, R. D. (1974) *J. Biol. Chem.* **249**, 4335–4338
- Holmberg, L., Lecander, I., Persson, B. & Åstedt, B. (1978) *Biochim. Biophys. Acta* **544**, 128–137
- Johansson, B. G. (1972) *Scand. J. Clin. Lab. Invest. Suppl.* **124**, 21–37
- Joist, J. H., Niewiarowski, S., Nath, N. & Mustard, J. F. (1976) *J. Lab. Clin. Med.* **87**, 659–669
- Laurell, C. B. (1972) *Scand. J. Clin. Lab. Invest. Suppl.* **124**, 21–37
- Mattler, L. E. & Bang, N. U. (1977) *Thromb. Haemostasis* **38**, 776–792
- McDonagh, J., Kiesselbach, T. H. & Wagner, R. H. (1969) *Am. J. Physiol.* **216**, 508–513
- Miletich, J. P., Jackson, C. M. & Majerus, P. W. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4033–4036
- Moore, S., Pepper, D. & Cash, J. D. (1975) *Biochim. Biophys. Acta* **379**, 360–369
- Moroi, M. & Aoki, N. (1976) *J. Biol. Chem.* **251**, 5956–5965
- Mui, P. T. K., James, H. L. & Ganguly, P. (1975) *Br. J. Haematol.* **29**, 627–637
- Müllertz, S. (1974) *Biochem. J.* **143**, 273–283
- Müllertz, S. (1978) *Prog. Chem. Fibrinolysis Thrombolysis* **3**, 213–237
- Müllertz, S. & Clemmensen, I. (1976) *Biochem. J.* **159**, 545–553
- Murray, J., Crawford, G. P. M. & Ogston, D. (1974) *Br. J. Haematol.* **26**, 661–668
- Nachman, R. L. & Harpel, P. C. (1976) *J. Biol. Chem.* **251**, 4514–4521
- Olsson, P. S. (1974) *Thromb. Res.* **5**, 1–18
- Østerud, B., Rapaport, S. I. & Lavine, K. K. (1977) *Blood* **49**, 819–834
- Ouchterlony, Ö. (1953) *Acta Pathol. Microbiol. Scand.* **32**, 231–240
- Thorell, J. I. & Johansson, B. G. (1971) *Biochim. Biophys. Acta* **251**, 363–369
- Thorsen, S. (1973) *Scand. J. Clin. Lab. Invest.* **31**, 51–59
- Thorsen, S., Brakman, P. & Astrup, T. (1972) *Hematol. Rev.* **3**, 123–179
- Vogel, R., Trautschold, I. & Werle, E. (1968) *Natural Proteinase Inhibitors*, pp. 95–107, Academic Press, New York and London
- Wakabayashi, K., Fujikawa, K. & Abe, T. (1970) *Thromb. Diath. Haemorrh.* **24**, 76–84
- Walsh, P. N. (1974) *Blood* **43**, 597–605
- Weber, K. & Osborn, M. (1975) *The Proteins* **1**, 179–223
- Wiman, B. & Wallén, P. (1973) *Eur. J. Biochem.* **36**, 25–31