Isolation and Characterization of a Human Plasma Protein with Affinity for the Lysine Binding Sites in Plasminogen

ROLE IN THE REGULATION OF FIBRINOLYSIS AND IDENTIFICATION AS HISTIDINE-RICH GLYCOPROTEIN*

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During chromatography of plasminogen- and fibrinogen-depleted human plasma on an insolubilized fragment of plasminogen which contains the high affinity lysine-binding site (LBS I-Sepharose), both α2-antiplasmin and another protein are bound and subsequently eluted with 10 mM 6-aminohexanoic acid. This other protein was purified to homogeneity by DEAE-Sephadex chromatography followed by immunoadsorption and obtained with a yield of 2.5 mg/liter of plasma. Alternatively, this protein was partially purified (≈90% pure) by chromatography on CM-cellulose followed by chromatography on LBS I-Sepharose and obtained with a yield of 40 mg/liter of plasma.

The purified protein was found to interact with the high affinity lysine-binding site in plasmin with an apparent dissociation constant of 0.9 μM, thereby markedly reducing the reaction rate between plasmin and α2-antiplasmin. The partially purified protein reacted similarly with an apparent dissociation constant of 2 μM. The purified protein caused a limited increase in the apparent dissociation rate of plasminogen by urokinase. In a 125I-fibrin system, the purified protein retarded fibrinolysis by a mixture of tissue plasminogen activator and plasminogen. This phenomenon may be explained by reduction of the apparent dissociation of plasminogen with the high affinity lysine-binding site available for binding to fibrin. The apparent dissociation constant of the interaction between plasminogen and the purified protein in this system was found to be 1.1 μM, which is in good agreement with that found between plasmin and the purified protein. The purified protein at a concentration of 1.8 μM reduced the binding of plasminogen to a fibrin clot by ≈90%.

The purified protein is a single-chain glycoprotein with an M₀ of 60,000 on SDS-gel electrophoresis and a NH₂-terminal sequence Val-Ser-Pro-. Its amino acid composition is apparently identical to that of a previously described plasma protein with an unknown biological function called histidine-rich glycoprotein (Heimburger, N., Haupt, H., Kranz, T., and Baudner, S. (1972) Hoppe-Seyler’s Z. Physiol. Chem. 353, 1133–1140) and the protein reacted with a monospecific antiserum against histidine-rich glycoprotein. Its concentration in plasma measured by electroimmunoassay is ≈1.8 μM.

Since the interaction between the high affinity lysine-binding site of plasminogen and the purified protein

Plasminogen contains structures, called lysine-binding sites, which specifically bind certain amino acids with antifibrinolytic properties, such as lysine, 6-aminohexanoic acid, and trans-4-aminomethylcyclohexane-1-carboxylic acid (1–3). These structures are involved in the interaction between plasmin(ogen) and fibrin (4–7) and between plasmin(ogen) and α2-antiplasmin (8–12). These lysine-binding sites play a crucial role in the regulation of physiological fibrinolysis (13). Plasminogen contains one lysine-binding site with high affinity (Kd = 9 μM) and four or five sites with low affinity (Kd = 5 mM) for 6-aminohexanoic acid (14); all of them are located in the plasmin A (heavy)-chain part of the molecule (15).

The specific interaction between the lysine-binding sites of plasminogen and α2-antiplasmin has been used for the isolation of the inhibitor by affinity chromatography on the insolubilized proenzyme (8, 9). An improved version of this affinity chromatography using an insolubilized fragment of plasminogen containing the high affinity lysine-binding site was recently announced and found to yield a purified inhibitor in a single-step procedure (16).

Using this method, however, we observed that besides α2-antiplasmin, another plasma protein was also bound to the insolubilized high affinity lysine-binding site of plasminogen and could be dissociated with 10 mM 6-aminohexanoic acid. This protein was further purified to homogeneity by DEAE-Sephadex chromatography and immunoadsorption. In the present study, this protein will be referred to as “the purified protein.” In view of its affinity for the lysine-binding site(s) of plasminogen, it might represent a physiological analogue of 6-aminohexanoic acid and therefore, its potential role in fibrinolysis was investigated. Physicochemical characterization revealed that this protein is a single-chain glycoprotein with a molecular weight of ≈60,000 and an unusually high histidine content. Its amino acid composition was found to be very similar to that of a previously described plasma protein with unknown biological function which was called histidine-rich.

1 In the present study, α2-antiplasmin designates the fast-acting plasmin inhibitor of human plasma, as suggested by the Subgroup on Inhibitors of the International Committee on Thrombosis and Haemostasis (1978) Thromb. Haemostasis. 39, 524–525.)
glycoprotein (17, 18). The identity of these two proteins was eventually confirmed by immunological cross-reactivity of an antiserum raised against histidine-rich glycoprotein with the protein purified in the present study.

MATERIALS AND METHODS

Plasminogen and Plasmin—Native plasminogen (NH2-terminal glutamic acid) was prepared from human plasma by affinity chromatography on lysine-Sepharose (19) followed by gel filtration on Ultrogel AcA 44. Activation to plasmin was performed with streptokinase (1000 IU/ml of plasminogen) in 0.1 M phosphate, pH 7.30, containing 25% glycerol at 0°C. The concentration was determined by active site titration with p-nitrophenyl-p'-guanidinobenzoate (20); at least 95% activation was obtained. The solution was stored in aliquots at −20°C until used.

The plasminogen fragment LBS F (triple-loop structures numbers 1 to 3 in the plasmin A-chain, Mr = 36,000) and low molecular weight plasminogen (triple-loop number 5 of the A-chain and the intact B-chain, Mr = 39,000) were obtained by elastase digestion of plasminogen followed by gel filtration on Sephadex G-75 and affinity chromatography on lysine-Sepharose essentially as described by Sottrup-Jensen et al. (21). Treatment of the low molecular weight plasminogen in the presence of the intact plasminogen yielded 55% activation to low molecular weight plasmin (low Mr-plasmin), as determined by active site titration with p-nitrophenyl-p'-guanidinobenzoate (20). Glu-plasminogen types I and II were obtained by chromatography on lysine-Sepharose, essentially as described by Brockway and Castellino (3).

α2-Antiplasmin—This was purified by affinity chromatography of human plasma on LBS 1-Sepharose (16), followed by chromatography on DEAE-Sephadex A-50. For the kinetic experiments, α2-antiplasmin was dissolved in 0.1 M sodium phosphate buffer, pH 7.30. Its activity was determined by titration against plasmin of known concentration (11) and amounted to 190%. The solution was stored in aliquots at −20°C until used.

Fibrinogen—Prepared from freshly frozen citrated plasma according to the method of Blomback and Blomback (22), the concentration was determined spectrophotometrically using A470 = 1.51 at 280 nm (22). It was labeled with fluorescein isothiocyanate (IRE, Fleurs, Belgium) using a reduced volume version (23) of the chloramine-T method (24).

Thrombin—Human thrombin was purified essentially as described by Fenton et al. (25). The concentration was determined spectrophotometrically using A115 = 18.0 at 280 nm (25).

Tissue Plasminogen Activator—Prepared from human uterine tissue as described (26), the activator was a kind gift from Dr. D. C. Rijken (Center for Thrombosis and Vascular Research, Leuven). Its activity was expressed in urokinase equivalent units by comparison of its fibrinolytic effect on plasminogen-enriched fibrin films.

Other Reagents—Urokinase was a kind gift from Dr. Sewell (Abbott, Chicago, IL). The plasmin substrate p-Val-Leu-Lys-Nan (S-2251, Coatest) and streptokinase (Kabi) were obtained from Kabi. Elastase was purchased from Sigma, p-nitrophenyl-p'-guanidinobenzoate and gelatin from Merck, 6-aminohexanoic acid from BDH, Sephadex products from Pharmacia, Ultrogel AcA 44 from LKB, and CM-cellulose (CM52) from Whatman.

Amino Acid Analysis—The samples were hydrolyzed with 6 M HCl in a vacuum at 110°C for 20 h. Analysis was performed using a Locarte amino acid analyzer.

NH2-terminal and COOH-terminal Amino Acid Sequence Determination—NH2-terminal sequence analysis was performed by manual Edman degradation (27). The phenylthiohydantoins were identified by thin layer chromatography (28) and quantitated spectrophotometrically (27). To confirm these results, the dansyl method of Gray and Hartley (29) was used.

The abbreviations used are: LBS 1, a fragment of plasminogen isolated after digestion with elastase, consisting of triple-loop structures ("kringles") 1 to 3 of the plasmin A-chain with an Mr of ~35,000; the rest of the (C-terminal) part of Tyr to Val 357, and Tyr 79 to Val 353 (21); low Mr-plasmin, a plasmin variant with an Mr of ~39,000, consisting of triple-loop structure 5 of the plasmin A-chain and an intact plasmin B-chain; it consists of Val 442 to Asn 790 (21); Glu-plasminogen, human plasminogen with NH2-terminal glutamic acid, required for protease activity; p-Val-Leu-Lys-Nan, S-2251, p-valyl-l-leucyl-l-lysyl-p-nitroanilide, a synthetic chromogenic substrate for plasmin (Kabi AB, Stockholm, Sweden).

COOH-terminal amino acids were determined by digestion with carboxypeptidase Y and carboxypeptidase A analysis. About 30 nmol (determined by reaction with ninhydrin after alkaline hydrolysis) was dissolved in 0.05 M sodium acetate buffer, pH 5.5, to a protein concentration of ~4 mg/ml. Digestion was performed at 25°C with an enzyme to substrate ratio of 1:200 (w/w) and sampling after a time period of 20 to 180 min. The sample was heated in a boiling water bath for 5 min to inactivate the enzyme and diluted with 0.2 M sodium citrate buffer, pH 1.9, for amino acid analysis.

Polyacrylamide Gel Electrophoresis—This was performed in the presence of sodium dodecyl sulfate using 7% polyacrylamide gels as described by Weber and Osborn (31).

Immunohistochemical Analysis—Double immunofluorescence was performed according to Ouchterlony (21) and electroimmunoassay according to Laurell (32). Rabbit antisera against human α2-antiplasmin and against the purified protein were prepared by injection of the purified antigens using the immunization scheme previously described (34). The antiserum against the histidine-rich glycoprotein of human plasma was a kind gift from Dr. Karges (Behringwerke AG, Marburg/Lahn, Federal Republic of Germany).

For immunoadsorption, the immunospecific immunoglobulins isolated from antisera against α2-antiplasmin were coupled to CNBr-activated Sepharose 4B. A column (1.6 × 20 cm) with a binding capacity of ~500 μg of α2-antiplasmin was prepared.

Determination of the Dissociation Constant of the Complex Between Plasmin and the Purified Protein—This dissociation constant was determined as the protein concentration that caused a decrease of the apparent rate constant (kapp) of the reaction between plasmin and α2-antiplasmin to 0, as previously described (35).

The purified protein was dissolved in different concentrations (0 to 5 μM) with the plasmin substrate S-2251 (final concentration, 0.6 mM) and plasmin was added to a final concentration of 8 nM. After a 30-s incubation, the change in absorbance at 410 nm was recorded for ~60 s, α2-antiplasmin was added to a final concentration of 12.5 nM, and the change in absorbance was again recorded. The apparent rate constant, kapp, was obtained from the residual plasmin activity at different time intervals using the classical second order rate equation. Plasmin activities were obtained from the initial slope of the curve (before 50% of the enzyme was inactivated).

The influence of the purified protein on the reaction between plasmin (final concentration, 25 nM) and α2-antiplasmin (final concentration, 115 nM) in the presence of 1 mM 6-aminohexanoic acid and on the reaction between low Mr-plasmin (final concentration, 15 nM) and α2-antiplasmin (final concentration, 115 nM) was also examined (pseudo-first order conditions) using the same procedure. Under these conditions, the rate of the reaction is decreased by about two orders of magnitude (11, 12).

All kinetic measurements were carried out with a Pye Unicam SP 1800 spectrophotometer in 0.1 M sodium phosphate buffer, pH 7.30, at 25°C, using 1.0 ml volumes in cuvettes with 1-cm path length. The Influence of the Purified Protein on the Activation of Plasminogen by Urokinase—Plasminogen was dissolved in phosphate-buffered saline containing 25% glycerol to a concentration of about 6 μM. In the absence or presence of different concentrations of the purified protein was performed by incubation of the mixture with urokinase (4500 IU/ml of plasminogen) at 25°C. At different time intervals, 10-μl samples were removed from the incubation mixture and plasmin was measured in a total volume of 1 ml of 0.1 M phosphate buffer, pH 7.30, using 0.3 mM S-2251. The experiments with Glu-plasminogen type I and II were performed under the same conditions.

Influence of the Purified Protein on the Activation of Plasminogen by Tissue Plasminogen Activator in the Presence of Fibrin—These experiments were performed on tissue culture plates (Falcon) as described (36), however, with the following modifications. Into each well of the plate (6-mm inner diameter) 100 μl of tissue plasminogen solution (4 × 103 cpm/ml) corresponding to 180 to 200 μg/ml was added and the plate was dried overnight at 37°C. Then, 75 μl of phosphate-buffered saline containing 3 μg of purified thrombin was added and the plates were incubated for 10 to 16 hours. Three aliquots of phosphate-buffered saline were then performed to remove thrombin. Before use, the plates were washed twice again to remove small amounts of soluble radioactive material.
Influence of the Purified Protein on the Binding of Plasminogen to Fibrin—These experiments were performed essentially as described by Rákóczi et al. (7). Mixtures of 0.5 ml of normal plasma or of plasma which was depleted of the purified protein by chromatography on CM-cellulose as described below, containing a trace amount of 125I-labeled Glu-plasminogen (about 60,000 cpm), were clotted by the addition of 0.4 NIH unit of thrombin. The fibrin threads were washed on a glass rod at 37°C during ~30 min and squeezed against the wall of the tube to express as much fluid as possible. The fibrin film was then washed twice by soaking it in 1 ml of 0.075 M NaCl, 0.05 M Tris-HCl buffer, pH 7.5, for 15 min at room temperature. Bound plasminogen was then eluted with the same buffer containing 20 mM 6-aminohexanoic acid.

Similar experiments were performed in a purified system. The clotting solution consisted of fibrinogen (final concentration, 6.3 μM), Glu-plasminogen containing 125I-labeled plasminogen (final concentration, 1.5 μM) or Glu-plasminogen preincubated with the purified protein (final concentration, 1.8 μM) and 0.4 NIH unit of thrombin in a total volume of 0.5 ml.

The amount of 125I-labeled plasminogen in the supernatant after clotting, in the wash buffer, in the eluates, and on the fibrin, was quantitated in a scintillation counter and expressed as a percentage of the original amount added to the plasma.

RESULTS

Isolation of "the Purified Protein"—Purification was performed using fibrinogen and plasminogen-depleted human plasma by affinity chromatography on LBS I-Sepharose and elution with 6-aminohexanoic acid, essentially as described by Wiman (16). Plasminogen was removed by chromatography on lysine-Sepharose and fibrinogen by precipitation with 10% ethanol at ~4°C. The materials obtained from 6 and 10 batches of plasma (which was depleted of fibrin) were dialyzed, lyophilized, and redissolved in a solution containing 0.04% azide and at pH 7.0, and applied to a DEAE-Sephadex A-50 column (5 × 20 cm) at 4°C with a flow rate of 30 ml/h. Elution was performed with a linear gradient of 5 column volumes of 0.05 M phosphate, pH 7.0, to 5 volumes of the same buffer containing 0.4 M NaCl at 60 ml/h.

The elution pattern (Fig. 1A) shows 2 major protein peaks. Total protein recovery from the ion exchange column was ~70%. Peak II contains the pure and fully active α2-antiplasmin as shown by Laurell electrophoresis, titration against plasmin of known concentration, amino acid analysis, and NH2-terminal sequence analysis. The same techniques indicated that Peak I contained an inactive form of α2-antiplasmin together with another component. The inactive α2-antiplasmin present in Peak I was removed by immunoadsorption. In one typical experiment, the protein (97 absorbance units at 280 nm) was eluted to the antibody column (1.6 × 20 cm) in 0.01 M phosphate buffer, pH 7.40, containing 0.14 M NaCl and 0.04% azide at 4°C and at a flow rate of 20 ml/h. Elution of α2-antiplasmin was performed with 0.1 M glycine-HCl, pH 2.80 (Fig. 1B). The filtrate through this column (Peak I A) contained the purified protein (23.5 absorbance units), while the eluate (peak IB) contained inactive α2-antiplasmin (7.5 absorbance units). Both were desalted by gel filtration on Sephadex G-25 in water. The protein recovery in this step was 84%. Assuming a protein concentration for the purified protein of 100 mg/liter (see below), the total yield in this procedure is only 2%. SDS-gel electrophoresis revealed a single band with an estimated molecular weight of ~60,000.

The complete removal of 6-aminohexanoic acid during this purification procedure was demonstrated by amino acid analysis. Therefore, we added 6-aminohexanoic acid (10% by weight) to our purified protein (1 mg/ml), the protein was precipitated with 20% CCl4COOH, and a sample of the supernatant (containing about 70 nmol of 6-aminohexanoic acid) was applied to an amino acid analyzer. 6-Aminohexanoic acid was eluted in a position between histidine and arginine with a recovery of 50%. A control sample of the purified protein alone treated in the same way did not reveal any traces of 6-aminohexanoic acid. The lower detection limit of our analyzer is about 0.2 nmol of amino acid, corresponding to less than 0.05% of 6-aminohexanoic acid in the purified protein.

Determination of the Dissociation Constant of the Complex between Plasmin and the Purified Protein—The influence of the purified protein on the apparent rate constant of the rapid reversible complex formation Kd,μ between plasmin and α2-antiplasmin is illustrated in Fig. 2. The purified protein induces a concentration-dependent reduction of this reaction rate. The shape of this sigmoidal curve is compatible with a single association reaction between plasmin and the purified protein. The dissociation constant of this interaction, determined as the concentration of purified protein which decreases the rate of the reaction between plasmin and α2-antiplasmin to 50% of its normal value, is 0.9 μM. Control experiments showed that addition of the purified protein to plasmin did not influence the rate of hydrolysis of S-2251.

Additional experiments showed no influence of the purified protein on the rate of the reaction between plasmin and α2-antiplasmin in the presence of 1 mM 6-aminohexanoic acid (which saturates the high affinity lysine-binding site), or on the rate of the reaction between low Mw-plasmin (which lacks the lysine-binding sites) and α2-antiplasmin (Fig. 2). All these findings indicate that the purified protein competes with α2-antiplasmin for the high affinity lysine-binding site in plasmin. Albumin did not interfere with the plasmin-α2-antiplasmin reaction in concentrations up to 0.1 mM.

Influence of the Purified Protein on the Activation of Glu-Plasminogen by Urokinase—Fig. 3 shows that the activation of Glu-plasminogen by urokinase is to some extent influenced by the presence of the purified protein. At a final concentration of 10 μM purified protein, a significant enhancement of the activation rate is observed. This influence increases to a final concentration of ~100 μM; a further increase to 400 μM only yields a minor additional change in the activation rate. The influence of the purified protein is not as pronounced as that obtained with 1 mM 6-aminohexanoic acid. A control experiment with 500 μM albumin did not show any increase in the activation rate of Glu-plasminogen.

The activation of Glu-plasminogen types I and II by urokinase was studied under the same experimental conditions in the absence and the presence of 60 μM purified protein. The activation rate of type II Glu-plasminogen was shown to be slightly slower than that of type I, but the effect of the purified protein was very similar to that observed with native Glu-plasminogen.

Influence of the Purified Protein on the Activation of Plasminogen by Tissue Activator in the Presence of Fibrin—Using mixtures of tissue plasminogen activator (final concentration was equivalent to 0.5 IU of urokinase) and different
plasminogen concentrations (0 to 500 nM), the rate of 
\(^{125}\)I release from 
\(^{125}\)I-fibrin plates increased with increasing plas-
minogen concentration (Fig. 4A). By plotting the time re-
quired to solubilize half of the radioactive fibrin (S,) versus
the logarithm of the plasminogen concentration, a straight
line is obtained in the range of 10 to 200 nM (Fig. 4B).

Using this system, we studied the influence of 6-aminohex-
anoic acid (0 to 40 \mu M) and the purified protein (0 to 20 \mu M)
on the digestion of 
\(^{125}\)I-fibrin by mixtures of human plasmin-
ogen (200 nM) and tissue plasminogen activator. The rate of
\(^{125}\)I release as a function of the concentration of 6-aminohex-
anoic acid or of the purified protein is shown in Fig. 5, A and
B, respectively. The displacement in the curves resulting in
higher S, values in the presence of 6-aminohexanoic acid or
the purified protein is compatible with the interpretation that
complex formation between plasminogen and 6-aminohexa-

![Fig. 1. Isolation of "the purified protein." A, elution pattern of the protein obtained from LBS I-Sepharose on
a DEAE-Sephadex A-50 column (5 x 20 cm) with a linear gradient of 0.05 M phosphate bufer, pH 7.0, to the same buffer
containing 0.4 M NaCl. The SDS-poly-
acylamide gels inserted show the prepara-
tion before applying to the column;
peak I (1), peak II (2), and peak III (3); gel electrophoresis was performed in the absence of reducing agents. B, chromatog-
raphy of I from DEAE-Sephadex on
anti-\(\alpha_2\)-antiplasmin-Sepharose (1.6 x 20 cm) in 0.01 M
phosphate buffer, pH 7.40, containing
0.14 M NaCl and 0.04% azide. The
arrow indicates the start of the elu-
tion of bound \(\alpha_2\)-antiplasmin with 0.1
M glycine-HCl, pH 2.80. The SDS-poly-
acylamide gels inserted show the puri-
fied protein of the first peak (1) and
inactive \(\alpha_2\)-antiplasmin of the second
peak (2).]
Antifibrinolytic Plasma Protein

Influence of the purified protein on the plasmin α2-antiplasmin reaction. Apparent rate constant of the reaction between plasmin and α2-antiplasmin in the absence (two independent series of experiments, ●, ○) or in the presence of 1 mM 6-aminohexanoic acid (□) and of the reaction between low Mr-plasmin and α2-antiplasmin (○).

Influence of the purified protein on the binding of plasminogen to fibrin in a purified system, in normal plasma, and in plasma depleted in the purified protein. Plasminogen concentration was 10 μM, and the fibrinogen concentration was 2.2 mg/ml. The depletion of the purified protein was achieved by adsorbing plasma twice with 50 g of CM-cellulose/liter at pH 6.0 after 2-fold dilution with distilled water. The plasma was then reconstituted to its original volume by ultrafiltration and the pH readjusted to 7.4. The concentration of the purified protein, measured by electroimmunoassay, was less than 10% of the original concentration. The fibrinogen concentration in the depleted plasma was reconstituted to its original value of 2.2 mg/ml by addition of 0.5 mg of purified fibrinogen/ml. The plasminogen concentration was unaltered by the adsorption with CM-cellulose.

In a purified system, the presence of the purified protein decreases the amount of Glu-plasminogen that specifically (eluted with 20 mM 6-aminohexanoic acid) binds to fibrin from 9.8 to 6.1%. In the purified protein-depleted plasma, 3.6% of Glu-plasminogen binds specifically to the fibrin clot compared to 2% in normal plasma. These findings support the concept that the purified protein reduces the binding of plasminogen to fibrin by complex formation with the lysine-binding site(s) of the proenzyme. The apparent dissociation constant of this interaction would be ~1 μM.
**Antifibrinolytic Plasma Protein**

**FIG. 5. Influence of 6-aminoheptanoic acid and the purified protein on the digestion of 125I-fibrin by mixtures of human plasminogen (200 nM) and tissue plasminogen activator (0.5 urokinase equivalent unit/ml).** A, cumulative release of 125I as a function of the concentration of 6-aminoheptanoic acid (indicated in μM). B, cumulative release of 125I as a function of the concentration of the purified protein (indicated in μM). C, plot of the apparent plasminogen concentration (in per cent of control value) versus the concentration of 6-aminoheptanoic acid (○) or purified protein (●). The apparent plasminogen concentration was determined from the S values using the calibration curve represented in Fig. 4B.

**TABLE I**

Influence of the purified protein on the adsorption of Glu-plasminogen to fibrin

| Sample                | Per cent bound to fibrin | Per cent eluted with 6-aminoheptanoic acid | Per cent remaining on fibrin |
|-----------------------|--------------------------|-------------------------------------------|-------------------------------|
| Glu-plasminogen       | 19.2 ± 1.5*              | 9.8 ± 0.6                                 | 9.4 ± 1.5                    |
| Glu-plasminogen with  | 11.4 ± 0.4               | 6.1 ± 0.6                                 | 5.3 ± 0.8                    |
| purified protein      |                          |                                           |                              |
| Normal plasma         | 4.5 ± 0.5                | 2.0 ± 0.4                                 | 2.5 ± 0.5                    |
| Purified protein-depleted | 9.5 ± 1.5              | 3.6 ± 0.4                                 | 5.8 ± 0.9                    |

* n, number of experiments.

The data represent mean ± S.D.

**TABLE II**

Amino acid composition of the purified protein

| Amino acid       | Mol of amino acid/mol of protein |
|------------------|----------------------------------|
| Aspartic acid    | 43                               |
| Threonine        | 14                               |
| Serine           | 29                               |
| Glutamic acid    | 42                               |
| Proline          | 50                               |
| Glycine          | 29                               |
| Alanine          | 18                               |
| Cysteine         | 15                               |
| Valine           | 18                               |
| Methionine       | 17                               |
| Isoleucine       | 8                                |
| Leucine          | 25                               |
| Tyrosine         | 10                               |
| Phenylalanine    | 22                               |
| Lysine           | 19                               |
| Histidine        | 54                               |
| Arginine         | 28                               |
| Tryptophan       | 2                                |

Physicochemical Characterization of the Purified Protein and Identification as Histidine-rich Glycoprotein—The results of amino acid analysis of the purified protein represented in Table II revealed a high histidine content and only traces of methionine. Tryptophan and cysteine were not determined.
This amino acid composition is very similar to that of a previously described plasma protein with unknown biological function, called histidine-rich glycoprotein (17, 18). In immunodiffusion, the purified protein reacted with the antiserum against histidine-rich glycoprotein, obtained from Behringwerke, but not with antiserum against α2-antiplasmin (Fig. 6A).

In Laurell immunoelectrophoresis, the antiserum against histidine-rich glycoprotein reacted with the purified protein and with a single component in human plasma (Fig. 6B). The plasma concentration, determined by electroimmunoassay, was about 100 mg/liter which is in accordance with the findings of Heimburger et al. (18).

Edman degradation revealed the NH₂-terminal sequence Val-Ser-Pro- with a recovery of 0.78 mol of Val/mol of protein. The sequence Val-Ser- was confirmed using the dansyl method. Digestion with carboxypeptidase Y revealed -Phe-Leu as COOH-terminal sequence (Table III).

**Partial Purification of the Histidine-rich Glycoprotein from Human Plasma and its Influence on the Reaction between Plasmin and α₂-Antiplasmin**—One liter of human plasma was diluted 2-fold with distilled water, the pH was adjusted to 6.0, and the mixture was stirred with 50 g of CM-cellulose 52 for 1 h at room temperature (17). The ion exchanger was removed, washed with water, and transferred to a column (2.5 × 20 cm). Elution with a linear gradient of 4 column volumes of water to 4 volumes of 0.5 M NH₄HCO₃ yielded one major protein peak (370 absorbance units at 280 nm). This pool was devoid of α₂-antiplasmin, plasminogen, and fibrinogen. It was dialyzed against 0.05 M phosphate buffer, pH 7.0, and applied to a column (2.5 × 40 cm) of LBS I-Sepharose (300 mg of insolubilized LBS I). The major part of the protein (228 absorbance units at 280 nm) was recovered by washing with 2 column volumes of 0.05 M phosphate buffer, pH 7.0. Elution with 10 mM 6-aminohexanoic acid yielded a single protein peak (35 absorbance units at 280 nm) containing 50% of the amount of histidine-rich glycoprotein applied to the column. After extensive dialysis and desalting on Sephadex G-25 to remove 6-aminohexanoic acid, ~50 mg of protein was obtained. SDS-gel electrophoresis revealed two protein bands (Fig. 7A). Analysis of the material by electroimmunoassay revealed that the histidine-rich glycoprotein accounted for about 80% of the total protein (Fig. 7B). Thus, the final recovery of histidine-rich glycoprotein in the partially purified preparation was 40%.

This partially purified protein decreased the rate of the reaction between plasmin and α₂-antiplasmin in a similar way as the highly purified protein used in our previous experiments. The apparent dissociation constant of the interaction between the histidine-rich glycoprotein and plasmin was found to be ~2 μM, assuming a purity of 80%. This value is comparable to that of 0.9 μM obtained for the highly purified protein. Control experiments showed no influence of the partially purified protein on the activity of α₂-antiplasmin or on the stability of plasmin.

**DISCUSSION**

During purification of α₂-antiplasmin from human plasma by affinity chromatography on an insolubilized fragment of

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**TABLE III**

| Amino Acid | Time (min) |
|------------|------------|
|            | 20         | 40 | 60 | 120 | 180 |
| Leucine    | 0.14       | 0.33 | 0.59 | 1.0 | 1.20 |
| Phenylalanine | 0.09       | 0.21 | 0.38 | 0.55 | 0.90 |
| Threonine  | 0.05       | 0.12 | 0.31 | 0.38 | 0.45 |
| Glycine    | 0.31       | 0.43 | 0.62 |     |     |

**Fig. 6.** Immunochemical analysis of the purified protein. A, immunodiffusion of the purified protein (1) and α₂-antiplasmin (2) against antiserum raised with histidine-rich glycoprotein (3) and with α₂-antiplasmin (4). B, Laurell immunoelectrophoresis with antiserum against histidine-rich glycoprotein. A, normal plasma (dilutions 1:1, 1:2, 1:4, and 1:8) and B, purified protein (0.1 mg/ml) (dilutions 1:1, 1:2, 1:4, and 1:8). Electrophoresis was performed at 80 V for 8 h.

**Fig. 7.** Analysis of partially purified histidine-rich glycoprotein. A, SDS-gel electrophoresis in the absence of reducing agents of the preparation obtained after chromatography on CM-cellulose 52 and LBS I-Sepharose. B, Laurell immunoelectrophoresis with antiserum against histidine-rich glycoprotein. A, normal plasma (dilutions 1:1, 1:2 and 1:4), B, purified protein of the first preparation (0.1 mg/ml) (dilutions 1:1, 1:2 and 1:4), and C, histidine-rich glycoprotein obtained from CM-cellulose (0.1 mg/ml) (dilutions 1:1, 1:2, and 1:4).
plasminogen which contains the high affinity lysine-binding site (LBS I), another protein is also bound and elutes with 10 mM 6-aminohexanoic acid. This protein was further purified to homogeneity by DEAE-Sephadex chromatography and immunoadsorption and was obtained in a yield of approximately 2.5 mg/liter of plasma.

The purified protein apparently interacts with one or more of the lysine-binding sites in plasminogen and in that respect could have similar properties to 6-aminohexanoic acid and related antifibrinolytic amino acids. Three properties of 6-aminohexanoic acid have been clearly delineated. One property is that in purified systems, saturation of the high affinity lysine-binding site in plasmin slows down the rate of its interaction with α2-antiplasmin ~50 times (11). In this respect, the purified protein resembles 6-aminohexanoic acid. It interacts with plasmin by an apparently single association reaction with a dissociation constant of 0.9 μM. That this interaction is indeed mediated through the lysine-binding site is substantiated by the finding that no effect was observed on the reaction between α2-antiplasmin and low molecular weight plasmin which lacks the lysine-binding sites (12, 21), or on the reaction between plasmin and α2-antiplasmin in the presence of 1 mM 6-aminohexanoic acid, which saturates the high affinity lysine-binding site (11). The dissociation constant of the complex between Glu-plasminogen and α2-antiplasmin is 4 μM (35) and between plasmin and α2-antiplasmin (with an additional interaction through the active site) is 0.2 nM (11). From these data, we have previously calculated that at the concentrations of plasminogen (1.5 to 2 μM) and α2-antiplasmin (total, 1 μM, of which ~½ with affinity for the lysine-binding sites in plasminogen (37)) which occur in plasma, about 30% of the α2-antiplasmin would occur reversibly complexed with plasminogen. From the dissociation constant between plasminogen and the purified protein (1.1 μM), which is in good agreement with that between plasmin and the purified protein (0.9 μM) we can estimate that at the concentrations of plasminogen (1.5 to 2 μM) and the purified protein (1.8 μM) which occur in plasma, about 50% of the proenzyme would circulate in association with this protein. This interaction between the purified protein and plasminogen would reduce the concentration of free plasminogen in blood and reduce the extent of complex formation between plasminogen and α2-antiplasmin by a factor of 2, thereby increasing the concentration of free inhibitor by about 15%.

Another property of antifibrinolytic amino acids is that they induce a conformational change in the plasminogen molecule which results in an enhanced activation rate by urokinase (5, 38–39). It was recently shown that at concentrations which saturate the low affinity lysine-binding sites, a conformational change occurs (40). The purified protein in concentrations above 10 μM and up to 500 μM had only a limited accelerating effect on the activation rate of plasminogen. This indicates either that the purified protein reacts only very weakly with the low affinity lysine-binding sites or that such interaction only leads to limited acceleration of the activation rate, possibly as a result of steric hindrance. From these findings, we extrapolate that the interaction between plasminogen and the purified protein in plasma would not lead to enhanced activation of plasminogen in plasma.

A third well delineated property of 6-aminohexanoic acid and its analogs is that they interfere with the binding of plasminogen to fibrin (6, 7). This interaction most probably constitutes the molecular basis of the antifibrinolytic effect of these compounds (13). In a fibrinolytic assay mixture composed of 125I-labeled fibrin, plasminogen and tissue plasminogen activator, 6-aminohexanoic acid induced a concentration-dependent prolongation of the time required to solubilize half of the fibrin. This effect is probably due to complex formation between the high affinity lysine-binding site and the ligand, resulting in abolishment of the interaction with fibrin. Indeed, an apparent reduction of the free plasminogen concentration to 50% was obtained at a concentration of 14 μM which is in good accordance with the previously determined dissociation constant of 9 μM (14). In this assay, the purified protein also induced a concentration-dependent decrease of the apparent free plasminogen concentration compatible with a single association reaction with an apparent dissociation constant of 1.1 μM. This value is in good agreement with that obtained for the interaction between plasmin and the purified protein. Thus, at the concentration of purified protein occurring in the blood, the effective plasminogen concentration would be reduced by 50%. It should be stressed that the measurements were performed with a plasminogen concentration of 200 nM which is ~10 times lower than that occurring in plasma. At such a high plasminogen concentration, the effect of 6-aminohexanoic acid or the purified protein would not be observed in our system. Still, there is ample clinical evidence that 6-aminohexanoic acid has antifibrinolytic properties and from the analogy in the results obtained with the purified protein, we extrapolate that it has a similar antifibrinolytic effect. This is further substantiated by the finding that the purified protein at a concentration of 1.8 μM reduces the binding of plasminogen to a fibrin clot by ~50%, as well in a purified system as in plasma. The observed phenomena cannot be explained by contamination of our purified protein with 6-aminohexanoic acid, since this would require a contamination exceeding 10% by weight. By amino acid analysis, we could not detect any 6-aminohexanoic acid in our purified protein (detection limit lower than 0.05% by weight).

In summary, our findings suggest that the purified protein might play a role in the regulation of fibrinolysis, mainly by interference with the binding of plasminogen to fibrin. This would result in retardation of fibrinolysis. The limited enhancing effect on the activation of plasminogen at very high concentrations is probably physiologically irrelevant. Physicochemical analysis revealed that the purified protein is a single-chain glycoprotein with a molecular weight of ~60,000, with NH2-terminal sequence Val-Ser-Pro- and COOH-terminal sequence -Phe-Leu. Both the amino acid composition and immunochemical analysis indicated that it is identical with a previously described protein with unknown biological function called histidine-rich glycoprotein (17, 18).

The concentration of the purified protein (histidine-rich glycoprotein) in plasma is ~100 mg/liter and the recovery in our first purification is only ~2%. The alternative partial purification procedure, also using LBS I-Sepharose, but in the absence of α2-antiplasmin and using less extensive washing, yielded the (partially purified) protein with the same properties in a yield of ~40%. The low yield of the purified protein in our isolation procedure is, therefore, most likely not due to selection of a specific molecular form of this protein which would represent only a small fraction of the total. This indicates that both α2-antiplasmin and the histidine-rich glycoprotein probably have a comparable affinity for LBS I-Sepharose. The greatly differing recovery of α2-antiplasmin (~28%) and the purified protein (~2%) in our first purification procedure could thus result from different rate constants in the dissociation reaction from the ligand.

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Antifibrinolytic Plasma Protein

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