Purification and Characterization of a Novel Inhibitor of Urokinase from Human Urine

QUANTITATION AND PRELIMINARY CHARACTERIZATION IN PLASMA*

(Received for publication, May 15, 1986)

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Urokinase-related proteins in human urine occur mainly as a 1:1 complex of urokinase with an inhibitor (Stump, D. C., Thienpont, M., and Collen, D. (1986) J. Biol. Chem. 261, 1267–1273). BALB/c mice were immunized with this urokinase-urokinase inhibitor complex and spleen cells fused with mouse myeloma cells, resulting in hybridomas producing monoclonal antibodies. Three antibodies reacting with the complex but not with urokinase were utilized to develop a sensitive (0.5 ng/ml) enzyme-linked immunosorbent assay for the urokinase inhibitor, which was used for monitoring its purification by chromatography on zinc chelate-Sepharose, concanavalin A-Sepharose, SP-Sepharose C-50, and Sephadex G-100.

A homogeneous glycoprotein of apparent M, 50,000 was obtained with a yield of 40 μg/liter urine and a purification factor of 320. One mg of the purified protein inhibited 35,000 IU of urokinase within 30 min at 37°C. This protein was immunologically related to both the purified urokinase-urokinase inhibitor complex and to the inhibitor portion dissociated from it by nucleophilic dissociation. It was immunologically distinct from all known protease inhibitors, including the endothelial cell-derived fast-acting inhibitor of tissue-type plasminogen activator, the placentally secreted inhibitor of urokinase and protease nexin. In electrophoresis the protein migrated with β-mobility. Inhibition of uroki-

nase occurred with a second order rate constant (k) of 8 x 10^3 M^-1 s^-1 in the presence of 50 IU of heparin/ml. The urokinase inhibitor was inactive towards single-chain urokinase-type plasminogen activator and plasmin, but it inhibited two-chain tissue-type plasminogen activator with a k below 10^3 M^-1 s^-1 and thrombin with a k of 4 x 10^4 M^-1 s^-1 in the presence of heparin.

The concentration of this urokinase inhibitor in plasma from normal subjects determined by immunoassay was 2 ± 0.7 μg/ml (mean ± S.D., n = 25). The protein purified from plasma by immunosorption had the same M,, amino acid composition, and immunoactivity as the urinary protein. Furthermore, when urokinase was added to plasma, time-dependent urokinase-urokinase inhibitor complex formation was observed at a rate similar to that observed for the inhibition of urokinase by the purified inhibitor from urine. This urokinase inhibitor, purified from human urine, most probably represents a new plasma protease inhibitor.

**EXPERIMENTAL PROCEDURES**

Materials

Human urokinase, single-chain urokinase-type plasminogen activator and urokinase-urokinase inhibitor complex were purified from human urine (7). Human tissue-type plasminogen activator (u-PA') was purified from cultured melanoma cells as described (8). Human thrombin (Topostasin®) was obtained from Roche. Human plasmin was produced by activation of human plasminogen with streptokinase (9). Chromogenic substrates pyroglutamyl-Gly-Arg-p-nitroanilide (S-2444), D-Val-Leu-Lys-p-nitroanilide (S-2251), D-Ile-Pro-Arg-p-nitroanilide (S-2288), and D-Phe-pipecolyl-Arg-p-nitroanilide (S-2238) were all from Kabi. Horseradish peroxidase-conjugated IgG was prepared according to Nakane and Kawai (10). p-Phenylenediamine was from Fluka. The International Reference Preparation for uroki-


*This study was supported by grants from the Geconcerteerde Onderzoeksacts en and the Fonds voor Geneeskundig Wetenschappelijk Onderzoek. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1The abbreviations used are: t-PA, tissue-type plasminogen activator; S-2444 or pyroglutamyl-Gly-Arg-p-nitroanilide, L-pyroglutamyl-glycyl-L-arginine-p-nitroanilide hydrochloride; S-2251 or D-Val-Leu-Lys-p-nitroanilide, H-D-Valyl-L-tyrosyl-L-valyl-L-lysyl-p-nitroanilide hydrochloride; S-2288 or D-Ile-Pro-Arg-p-nitroanilide, H-D-isoleucyl-L-prolyl-L-arginine-p-nitroanilide dihydrochloride; S-2238 or D-Phe-pipecolyl-Arg-p-nitroanilide, H-D-phenylalanyll-L-pipecolyl-L-argi

nine-p-nitroanilide dihydrochloride; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PEG, polyethylene glycol.
(11). Concanavalin A-Sepharose, SP-Sephadex C-50, Sephadex G-100 superfine, protein A-Sepharose, and cyanogen bromide-activated Sepharose 4B were from Pharmacia P-L Biochemicals. All other chemicals and reagents were of standard laboratory grade.

Rabbit antibodies to the urinary urokinase inhibitor were prepared as follows. New Zealand White rabbits were injected subcutaneously with 100 μg of protein purified from urine, in complete Freund's adjuvant, followed by two equal doses in incomplete Freund's adjuvant at biweekly intervals. Immune serum was obtained 7–10 days after the third injection, and IgG was prepared by chromatography on protein A-Sepharose (12). Monospecific anti-urokinase inhibitor antibodies were obtained by chromatography of the immune IgG fraction on a 3-ml column of purified urokinase inhibitor coupled to cyanogen bromide-activated Sepharose 4B (0.5 mg/ml swelling gel). The affinity-purified anti-urokinase inhibitor IgG eluted from the column with 0.15 M NaCl, 0.1 mM glycin-HCl buffer, pH 2.2, and the pH was immediately adjusted to 7.8 by the dropwise addition of 1.0 M Tris-HCl buffer, pH 9.0. After overnight dialysis against 0.15 M NaCl, 0.02 M NaH\(_2\)PO\(_4\) buffer, pH 7.4, the purified antibodies were stored frozen at −20°C.

Monoclonal antibodies to the urokinase-urokinase inhibitor complex were prepared essentially as previously described (7) using the method of Galfre and Milstein (13) with P3X63-Ag8.65.3 mouse myeloma cells obtained from Dr. E. Schonherr, Organon, Oss, The Netherlands. Hybridomas were screened for antibodies against the urokinase-urokinase inhibitor complex that did not react with urokinase as follows. Microtiter plates (Titertek), coated with either urokinase-urokinase inhibitor complex or urokinase, were incubated with conditioned medium, and bound antibody was quantitated with peroxidase-conjugated rabbit anti-mouse IgG. Positive wells were subcloned by limiting dilution, and purified antibodies were isolated from ascites obtained after injection of hybridoma cells into pristane-primed mice by chromatography on protein A-Sepharose (12) and elution with glycine-HCl buffer, pH 2.2. From a total of seven antibodies, reacting specifically with the urinary urokinase-urokinase inhibitor complex but not with urokinase, three (11D4H7, 7B5F7, and 10B5D10) were used to develop the ELISA for the urokinase inhibitor, and one (10A6A10) was used for immunoadsorption chromatography of urokinase (401J, 6E8, and 10D3G10) were prepared and characterized as described (14).

Rabbit antisera to human α\(_2\)-macroglobulin, antithrombin III, and α\(_1\)-antitrypsin were obtained from Behringwerke (Marburg/Lahn, West Germany). Rabbit antisera to human α\(_2\)-antiplasmin were produced as described (15). Rabbit antiserum against the human placental troponin I was a gift from Dr. E. Redd (7).

Methods

Immunoabsorption of the Urokinase Inhibitor from Urine on an Insolubilized Monoclonal Antibody—A monoclonal antibody reacting with the urokinase-urokinase inhibitor complex but not with urokinase was coupled to cyanogen bromide-activated Sepharose 4B (2 mg/ml gel) as described above. One liter of fresh human urine was collected at 5 × 24-h intervals. Immune serum was obtained 7–10 days after the first immunization, as measured with the ELISA described above, was pooled with 100 μl of protein purified from urine, in complete Freund's adjuvant in the presence of the chromogenic substrate S-2251 (7) or in a fibrin-clot lysis assay previously described (17).

Enzyme-linked Immunosorbent Assay (ELISA) for the Urokinase Inhibitor—Three monoclonal antibodies reacting with the urokinase-urokinase inhibitor complex but not with urokinase were identified as reacting with distinct antigenic epitopes on the complex during double immunodiffusion as described by Ouchterlony (16). Two of these (11D4H7 and 7B5F7) were diluted at 0.01 M carbonate buffer, pH 9.6, to a final concentration of 5 μg/ml each, and then 200 μl were incubated in each of 96-well polystyrene microtiter plates for 72 h at 4°C. The plates were then emptied and remaining active sites were blocked by incubation with 0.14 M NaCl, 0.01 M NaH\(_2\)PO\(_4\), pH 7.2, containing 0.01% Tween 80 and 1% bovine serum albumin for 1.5 h at 37°C. The plates were then washed with the same buffer without albumin just prior to use. Plates to be stored were incubated with 200 μl/well of buffer containing 100 μg of mannitol and 20 g of sucrose/liter for 3 min at room temperature, emptied, covered, and kept at −20°C. Just before use, each plate was washed three times with phosphate buffer.

Test samples or calibration mixtures were incubated in 180-μl aliquots/well for 2 h at 37°C or overnight at 4°C. After washing the plates six times with phosphate buffer, 170 μl of a third antibody (10B5D10) conjugated with horseradish peroxidase was added at a concentration of 0.09 μg/ml in a 0.1 M phosphate buffer containing 0.003% hydrogen peroxide, was added per well. After 30 min at room temperature the reaction was stopped with 50 μl of 4 M H\(_2\)SO\(_4\), and the absorbance was measured at 492 nm with a multiscan spectrophotometer (Titertek).

Purification of the Urokinase Inhibitor from Human Urine—Freshly voided urine from male laboratory personnel was collected at 5 × 24-h intervals. Blood was collected at 5 × 24-h intervals on benzamidine (5 mM final concentration) and accumulated for no longer than 6 h. The urine was then cooled by immersion in ice-water bath, after which all subsequent steps were carried out at 4°C. The urine was adjusted in pH to 7.5 with NaOH and centrifuged at 6000 × g for 30 min at 4°C. The supernatant was decanted and applied to a 5-ml column of 10A6A10-Sepharose at a flow rate of 10 ml/h at 4°C. After loading, the column was washed first with 10 volumes of 0.15 M NaCl, 0.02 M NaH\(_2\)PO\(_4\) buffer, pH 7.5, containing 5 mM benzamidine, and then with the same buffer without benzamidine until the absorbance at 280 nm was below 0.05 (5 to 10 column volumes). The column was then eluted with the same buffer containing 0.05 M imidazole, collecting 20-ml fractions. Fractions containing protein were pooled (usually about 1.5 ml) and concentrated to 2 ml by dialysis against 0.15 M NaCl, 0.02 M NaH\(_2\)PO\(_4\) buffer, pH 7.5, containing 5 mM benzamidine, stirred, and washed with an additional 2 liters of the same buffer. The column was then eluted with the same buffer containing 0.05 M imidazole, collecting 20-ml fractions. Fractions containing protein were pooled (usually about 8 ml) and dialyzed against 0.02 M NaCl, 0.02 M NaH\(_2\)PO\(_4\) buffer, pH 6.8, to a conductance equal to that of the dialysis buffer. The dialyzed sample was then applied to a 0.9 × 13-cm column of SP-Sephadex C-50, equilibrated with dialysis buffer, at a flow rate of 5 ml/h. The column was washed with the same buffer until the absorbance at 280 nm was less than 0.05 and then a linear 60-ml NaCl gradient from 0.02 to 0.5 M was applied. Two-mg fractions, containing urokinase inhibitor-related antigen, as measured with the ELISA described above, were pooled (usually about 20 ml) and concentrated to 2 ml by dialysis against 0.02 M NaCl, 0.02 M NaH\(_2\)PO\(_4\) buffer, pH 7.5, containing 5 mM benzamidine, and lyophilized. The lyophilized sample was then applied to a 2 × 8.5-cm column of concanavalin A-Sepharose equilibrated with 0.15 M NaCl, 0.02 M NaH\(_2\)PO\(_4\) buffer, pH 7.5, containing 5 mM benzamidine. The column was then eluted with the same buffer until the absorbance at 280 nm was less than 0.05 and then a linear 60-ml NaCl gradient from 0.02 to 0.5 M was applied. Two-mg fractions, containing urokinase inhibitor-related antigen, as measured with the ELISA described above, were pooled (usually about 80 ml), dialyzed against 0.02 M NaCl, 0.02 M NaH\(_2\)PO\(_4\) buffer, pH 6.8, to a conductance equal to that of the dialysis buffer. The dialyzed sample was then applied to a 0.9 × 13-cm column of SP-Sephadex C-50, equilibrated with dialysis buffer, at a flow rate of 5 ml/h. The column was washed with the same buffer until the absorbance at 280 nm was less than 0.05 and then a linear 60-ml NaCl gradient from 0.02 to 0.5 M was applied. Two-mg fractions, containing urokinase inhibitor-related antigen, as measured with the ELISA described above, were pooled (usually about 80 ml), dialyzed against 0.02 M NaCl, 0.02 M NaH\(_2\)PO\(_4\) buffer, pH 7.5, containing 5 mM benzamidine, and lyophilized. The lyophilized sample was then applied to a 1.5 × 90-cm column of Sephadex G-100 superfine at a flow rate of 4 ml/h. Two-mg fractions were assayed for both urokinase inhibitor-related antigen and urokinase inhibitory activity, and enriched fractions were pooled for further analysis.

Urokinase Inhibitor from Human Plasma—Blood samples were collected by venipuncture in 0.1 volume of 0.11 M citrate, and plasma was obtained following centrifugation at 3000 × g for 10 min at 4°C. Five hundred ml of plasma were applied at 20
ml/h at 4 °C to a 10-ml column of monoclonal antibody 10A6A10-Sepharose prepared as above, which was equilibrated with 0.15 M NaCl, 0.02 M NaH₂PO₄ buffer, pH 7.5, containing 0.01 M sodium citrate. After loading, the column was washed with the same buffer until the absorbance at 280 nm was below 0.05. The column was then eluted with 2 M KSCN and protein-containing fractions were pooled and dialyzed against column buffer. The dialyzed pool was then reapplied to a 2-ml column of 10A6A10-Sepharose at a flow rate of 4 ml/h, followed by a buffer wash and 2 M KSCN elution. Protein-containing fractions were pooled (generally about 5 ml), concentrated against solid PEG to 2 ml, dialyzed against gel filtration buffer, and applied to a Sephadex G-100 superfine column as described above. Fractions enriched in urokinase inhibitor-related antigen as measured by ELISA were pooled for further analysis.

Characterization of the Urokinase Inhibitor—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% slab gels according to Laemmli (17). Reduction of disulfide bonds was achieved with 0.05 M dithioerythritol and staining with Coomassie Brilliant Blue. Immunodiffusion analysis was performed according to Ouchterlony (16). Immunoblotting was performed after transferring protein from SDS-PAGE gels to nitrocellulose sheets by the method of Towbin et al. (18). The sheets were blotted with monospecific rabbit IgG antibodies (20 μg/ml) to either urokinase or urinary urokinase inhibitor, as previously described (7). Crossed immunoelectrophoresis using monospecific rabbit IgG against the urokinase inhibitor was performed according to Clarke and Freeman (19). Amino acid analysis was performed with a Beckman 119CL amino acid analyzer after sample hydrolysis with 6 M HCl in vacuo at 110 °C for 22 h. Inhibition rates for urokinase were measured by determining residual activity at timed intervals with S-2444 as described above. Inhibition rates were similarly measured for plasminogen (5 nM) with S-2251 (0.6 mM), for two-chain t-PA (9 nM) with S-2288 (1 mM), and for thrombin (1.2 mM) with S-2238 (0.1 mM). Inhibition rates for single-chain urokinase-type plasminogen activator were measured by determining residual plasminogen activation rates in the presence of excess S-2251 (1 mM).

ELISA for Urokinase-Urokinase Inhibitor Complex in Plasma—Microtiter plates were coated with two monoclonal antibodies (4D1E8 and 10D3G10) as described (14). Remaining active sites were blocked with 1% bovine serum albumin and the plates were stored in the same manner as described above. After washing with phosphate buffer, test samples or calibration mixtures containing purified urokinase-urokinase inhibitor complex were incubated in 180-μl volumes either for 2 h at 37 °C or overnight at 4 °C. After washing, the plates were incubated with the same horseradish peroxidase-conjugated monoclonal antibody to the urokinase inhibitor (10BS5D10) utilized in the ELISA for urokinase inhibitor described above. Quantitation of the bound conjugate was performed in the same manner with o-phenylenediamine.

RESULTS

ELISA for the Urokinase Inhibitor—The ELISA for the urokinase inhibitor was based on three monoclonal antibodies directed against nonoverlapping epitopes of the non-urokinase portion of the urokinase-urokinase inhibitor complex. It measured both the complex and the subsequently purified urokinase inhibitor similarly in a concentration range of 4 to 40 μM (0.5 to 5 ng/ml inhibitor), with a linear dose response (Fig. 1A). When either pooled human urine and citrated plasma were assayed, a linear dose response between 0 and 1% (v/v) for urine and between 0 and 0.1% (v/v) for plasma was obtained. When ultimately calibrated against known concentrations of purified urinary urokinase inhibitor, the concentrations in these individual samples were determined to be 0.15 and 3 μg/ml, respectively. The assay could be performed in one working day, which made its use to monitor the purification of the urokinase inhibitor from human urine practical.

Purification of the Urokinase Inhibitor from Urine—Initial purification of the urokinase inhibitor was performed by chromatography of fresh urine on the insolubilized monoclonal antibody 10A6A10. Material eluted from this column with 2 M KSCN contained a major protein band migrating with an apparent Mr of 50,000 on SDS-PAGE and in addition several minor high Mr bands (not shown). However, this material was inactive, presumably due to exposure to denaturants during its elution from the immunoadsorption column.

Therefore, an alternative purification procedure was developed in which the chromatographic behavior of the inhibitor was monitored with the ELISA described above. The results are summarized in Table I. Fresh human urine was collected on 5 mM benzamidine to inhibit proteolytic activity and to prevent further complex formation of urinary serine proteases with the urokinase inhibitor. Starting urokinase inhibitor-related antigen concentrations in urine were variable but generally averaged 200 ng/ml. In the first step, chromatography of cooled, pH-adjusted, human urine on zinc chelate-Sepharose resulted in 75% adsorption of urokinase inhibitor-related antigen. Elution with imidazole resulted in a recovery of 50% of the original antigen with a 30-fold volume reduction and a 10-fold purification. Direct application of this eluate to concanavalin A-Sepharose gave 90% adsorption of urokinase

![Fig. 1. Dose response of urokinase inhibitor in the enzyme-linked immunosorbent assay. A, absorbance at 492 nm with increasing concentrations of urokinase-urokinase inhibitor complex (C) and of purified urokinase inhibitor (●); B, absorbance of 492 nm with increasing volume fractions of pooled human urine (C) or citrated plasma (●).](image-url)
Urinary Urokinase Inhibitor

Characterization of the Urokinase Inhibitor from Urine—The purified urokinase inhibitor was analyzed by double immunodiffusion as shown in Fig. 5. It did not cross-react with antibodies to urokinase (A, spot 2), which reacted both with purified urokinase (A, spot 4) and with urokinase-urokinase inhibitor complex (A, spot 3). Monospecific antibodies isolated from the serum of rabbits immunized with the purified urokinase inhibitor reacted both with the urokinase inhibitor (B, spot 2) and the urokinase-urokinase inhibitor complex (B, spot 3) but not with urokinase (B, spot 4), suggesting that the purified urokinase inhibitor is identical to the protein which forms complexes with urokinase in urine. This was confirmed by immunoblotting of proteins transferred to nitrocellulose from reduced SDS gels similar to those of Fig. 4B. As shown in Fig. 6, both the urokinase-urokinase inhibitor complex (A, lane 1) and its dissociated subunits (A, lane 2) were all reactive with rabbit antibodies to urokinase except for the M₄, 50,000 nonreducible component of the digest. This component did, however, react with rabbit antibodies to the urinary urokinase inhibitor (B, lane 2) similarly to the urokinase-urokinase inhibitor complex (B, lane 1) and the urokinase inhibitor itself (B, lane 3). This establishes that the purified urokinase inhibitor is indeed the protein which forms a complex with urokinase in urine.

The purified urokinase inhibitor did not cross-react with antisera to the known plasma protease inhibitors antithrombin III, α₁-antiplasmin, α₁-antitrypsin, or α₂-macroglobulin. In addition, it did not react with antisera to the urokinase inhibitor from human placenta, protease nexin, or the endothelial fast-acting t-PA inhibitor. Furthermore, testing against a broad panel of antisera to 52 known plasma proteins did not result in a positive identification.

Crossed immunoelectrophoresis (Fig. 7) using monospecific antibodies to the urokinase inhibitor showed that it migrated as a β-globulin (Fig. 7A) when compared to normal plasma (Fig. 7B). Crossed immunoelectrophoresis of total human plasma using antibodies to the urokinase inhibitor did not

### Table I

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume</th>
<th>Total Protein</th>
<th>Total Urokinase Inhibitor</th>
<th>Total Urokinase Inhibitor/Total Protein</th>
<th>Yield (%)</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>24,000</td>
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<td>4.9</td>
<td>0.0032</td>
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<td>1</td>
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<tr>
<td>Zinc chelate-Sepharose</td>
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<td>73</td>
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<td>49</td>
<td>10</td>
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<td>1.6</td>
<td>0.81</td>
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<td>260</td>
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<td>Sephadex G-100</td>
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<td>0.9</td>
<td>0.9</td>
<td>1.0</td>
<td>18</td>
<td>320</td>
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</tbody>
</table>

* Determined by ELISA.

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2. D. Loskutoff, personal communication.
FIG. 3. Gel filtration pattern of protein, urokinase inhibitor-related antigen, and urokinase inhibitor activity on Sephadex G-100 superfine. 2 ml of concentrated material, eluted from SP-Sephadex C-50, was applied to a 180-ml column which was developed at a flow rate of 4 ml/h. 2-ml fractions were collected. —, absorbance at 280 nm; ○—O, urokinase inhibitor-related antigen; X—X, urokinase inhibitory activity. Fractions 24 to 38 were pooled. The column was calibrated using a mixture of phosphorylase b (M, 94,000), bovine serum albumin (M, 67,000), ovalbumin (M, 43,000), and soybean trypsin inhibitor (M, 20,000). The arrows indicate elution positions of the calibration proteins, expressed in kilodaltons.

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A, without reduction; B, after reduction with dithioerythritol. Lane 1, purified urokinase-urokinase inhibitor complex from urine; lane 2, urokinase-urokinase inhibitor complex after digestion with 0.5 M hydroxylamine; lane 3, purified urokinase inhibitor from urine; lane 4, purified urokinase inhibitor obtained by immunoabsorption of plasma; lane 5, M, 54,000 urokinase. Molecular weight calibration was performed with a standard mixture of phosphorylase b (M, 94,000), bovine serum albumin (M, 67,000), ovalbumin (M, 43,000), carbonic anhydrase (M, 30,000), and soybean trypsin inhibitor (M, 20,000).

Fig. 5. Double immunodiffusion. A, rabbit anti-urokinase IgG; B, rabbit anti-urokinase inhibitor IgG. Spot 1, urokinase inhibitor isolated from plasma by immunoabsorption (Fig. 4, lane 4); spot 2, urokinase inhibitor purified from urine (Fig. 4, lane 3); spot 3, urokinase-urokinase inhibitor complex (Fig. 4, lane 1); spot 4, M, 54,000 urokinase (Fig. 4, lane 5).

The amino acid composition of the urokinase inhibitor is shown in Table II. Only 0.6 residue of cysteine/100 residues was obtained, as determined both as cysteic acid or as carbamylcysteine. This suggests that the urokinase inhibitor contains 2 cysteine residues. Significant amounts of amino sugars were recovered, confirming its glycoprotein nature.

One mg of the purified urokinase inhibitor neutralized 35,000 IU of urokinase when incubated for 30 min at 37 °C in the presence of 50 units of heparin/ml. This suggested that only about 1/5 of the purified material from urine was obtained in functional form. Incubation of 10 nM urokinase with 70 to 280 nM active purified inhibitor (pseudo-first order conditions) led to a time and concentration-dependent loss of urokinase activity (Fig. 8A).

From Fig. 8A an apparent second order rate constant of 8

yield a precipitin arc (not shown), suggesting that the inhibitor occurs at a low concentration in plasma.
Urinary Urokinase Inhibitor

Isolation of the Urokinase Inhibitor from Human Plasma—When normal human plasma samples were assayed for urokinase inhibitor-related antigen by the ELISA calibrated with the urinary protein, a level of $2.0 \pm 0.75 \mu g/ml$ (mean $\pm$ S.D., $n = 25$) was found. This antigen was quantitatively removed by immunoadsorption on the insolubilized monoclonal antibody 10A6A10, a different antibody from those used in the ELISA. When plasma was subjected to two cycles of immunoadsorption and then to Sephadex G-100 gel filtration, a single polypeptide was isolated. This protein comigrated with the urokinase inhibitor purified from urine on gel filtration (not shown) and on SDS-PAGE (Fig. 4, A and B, lanes 4). It was immunologically identical to the purified protein from urine (Fig. 5B, lane 1) and reacted with monospecific IgG against the urokinase inhibitor during immunoblotting (Fig. 6B, lane 4). The amino acid composition of the material isolated from plasma was similar to that of the inhibitor isolated from urine (Table II). The protein isolated from plasma, however, did not inhibit urokinase, a finding which was also obtained with the urinary material when obtained by immunoadsorption. The urokinase inhibitor concentration in serum was the same as that in plasma.

![Crossed immunoelectrophoresis](image)

**Fig. 7.** Crossed immunoelectrophoresis. Three-ml samples were applied at the origins (o) and the tracking dye (bromphenol blue) was run to the marker (vertical bars in A and B). Rabbit antibodies to the urokinase inhibitor (0.5 mg) and rabbit antiserum to total human serum. Antiserum was added to the gel for electrophoresis in the second dimension. A, purified urinary urokinase inhibitor (0.5 mg) and rabbit antibodies to the urokinase inhibitor; B, total human serum, 4-fold diluted, and rabbit antiserum to total human serum.

**TABLE II**

<table>
<thead>
<tr>
<th>Amino acid composition of the urokinase inhibitor</th>
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<tbody>
<tr>
<td><strong>Amino acid</strong></td>
</tr>
<tr>
<td>Aspartic acid</td>
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<tr>
<td>Threonine</td>
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<tr>
<td>Serine</td>
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<tr>
<td>Glutamic acid</td>
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<td>Proline</td>
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<td>Glycine</td>
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<td>Alanine</td>
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<td>Arginine</td>
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<td>Tryptophan</td>
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<tr>
<td>Glucosamine</td>
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<td>Galactosamine</td>
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</table>

$^a$ The values represent mean of three determinations and are expressed as number of residues/100 residues of amino acids, not including tryptophan.

$^b$ Determined after conversion to cysteic acid or to carboxymethylcysteine.

$^c$ ND, not determined.

$\times 10^3$ M$^{-1}$ s$^{-1}$ was calculated. However, when heparin was added to the reaction mixture a more rapid initial inhibition rate with an apparent second order rate constant of $9 \times 10^4$ M$^{-1}$ s$^{-1}$ was seen (Fig. 8B). A similar effect of the urokinase inhibitor towards urokinase was observed in a clot lysis system where residual urokinase activity was measured via plasminogen activation rather than by its amidolytic activity (not shown). The protease specificity of the urokinase inhibitor was apparent by lack of inhibition of plasmin at 10-fold molar excess and of single-chain urokinase-type plasminogen activator at 4-fold molar excess of inhibitor. In addition, two-chain t-PA was inhibited 10-fold more slowly than urokinase. However, thrombin was inhibited with an apparent second order rate constant of $4 \times 10^4$ M$^{-1}$ s$^{-1}$ in the absence of heparin and $2 \times 10^5$ M$^{-1}$ s$^{-1}$ in the presence of 50 IU/ml of heparin. The urokinase inhibitory activity was stable during freezing and thawing but decreased rapidly over a few days if stored at 4°C. Urokinase inhibitory activity was stable for over 1 h at pH 4 but was completely destroyed within 5 min at pH 2.5. The inhibitory activity was also destroyed during a 1-h incubation in 0.1% SDS.

**Fig. 8.** Inhibition of urokinase by the urokinase inhibitor isolated from urine. Urokinase (10 nM) was incubated with bovine serum albumin (1 mg/ml) and purified active urokinase inhibitor (0.7 nM; C, 140 nM; X, 280 nM) at 37°C. At the times shown, aliquots were removed and diluted 9-fold with 0.038 M NaCl, 0.04 M Tris-HCl, pH 7.4, containing 0.01% Tween 80 and 0.33 mM S-2444. Residual urokinase activity was determined by measurement of absorbance at 405 nm as compared to controls without added urokinase inhibitor (milliabsorbance units/min = 0.011). A, without heparin; B, with heparin, 50 units/ml. Data points are expressed as percent of control urokinase activity.

![Graph](image)
Urokinase-Urokinase Inhibitor Complex Formation in Plasma—To determine if urokinase-urokinase inhibitor complexes form in human plasma following addition of urokinase, an ELISA specific for the purified complex from human urine was developed. It was based on the adsorption of urokinase-related antigen to microtiter plates coated with two monoclonal antibodies to urokinase, followed by quantitation of bound urokinase-urokinase inhibitor complex with a peroxidase-conjugated monoclonal antibody specific for the urokinase inhibitor. Purified urokinase-urokinase inhibitor complex could not be measured in undiluted plasma due to high background levels, but, when added to 10-fold diluted plasma, a linear dose response between 2 and 20 ng/ml was obtained (not shown). Thus, the effective sensitivity of this assay for this complex in plasma was 20 ng/ml (or 10 ng/ml of complex-associated urokinase antigen).

No urokinase-urokinase inhibitor complexes could be measured in normal resting plasma. However, when urokinase was added to plasma at a final concentration of 2 ng/ml, the assay measured time-dependent formation of this complex as shown in Fig. 9. In the absence of heparin the rate of complex formation was relatively slow and incomplete. However, when plasma contained 50 IU/ml heparin, both more rapid and more extensive complex formation occurred, reaching half-maximum levels in about 5 min and maximum levels after 20 to 30 min. Complex formation was not observed when urokinase with an active site blocked by the synthetic inhibitor Glu-Gly-Arg-CH2Cl was added to normal plasma, nor when active urokinase was added to plasma depleted of the urokinase inhibitor by immunoadsorption on insolubilized monoclonal antibody 10A6A10. From the initial rates of complex formation measured with enzyme and inhibitor concentrations of 40 nM each, second order rate constants of approximately $7 \times 10^8$ M$^{-1}$ s$^{-1}$ in the absence and $5 \times 10^4$ M$^{-1}$ s$^{-1}$ in the presence of heparin were calculated.

DISCUSSION

Our previous observation that urokinase is recovered from human urine mainly as a complex with an inhibitor (7) prompted us to purify and characterize this urokinase inhibitor. First, an immunoadsorbent useful for the monitoring of this inhibitor during purification was developed. Therefore, mice were immunized with the purified urokinase-urokinase inhibitor complex and hybridomas secreting monoclonal antibodies against epitopes localized in the inhibitor portion of the complex were identified. A rapid and sensitive ELISA for the urokinase inhibitor was developed, using three monoclonal antibodies reacting with three nonoverlapping epitopes in the inhibitor. Although highly purified urokinase inhibitor could be obtained by immunoadsorption chromatography of urine on insolubilized monoclonal antibody, this material was totally inactive. Therefore, an alternative purification method was developed which did not require the use of denaturing solvents.

With a concentration of urokinase inhibitor-related antigen in urine of 200 ng/liter, large volumes of urine were required to allow isolation of sufficient amounts of urokinase inhibitor for further characterization. Zinc chelate-Sepharose was chosen as the first step because this allows rapid handling of large volumes of urine. Although the extent of adsorption of the inhibitor to this column was only 75%, the 30-fold volume reduction and 10-fold purification were very useful. In the next step chromatography on concanavalin A-Sepharose yielded separation from free urokinase antigen, which passed unbound through the column, and provided further volume reduction and purification. Final purification was then obtained with both cation-exchange and gel filtration chromatography, yielding homogenous material, 320-fold purified, with a yield of 18% of the original starting material.

The purified urokinase inhibitor is a glycoprotein of apparent M, 50,000 with β-globulin mobility on electrophoresis. It is immunologically distinct from all known plasma protease inhibitors, including the fast-acting t-PA inhibitor (20), protease nexin (21), or the urokinase inhibitor from human placenta (22). Moreover, it does not react with a broad panel of antisera against known plasma proteins. Therefore, this urokinase inhibitor probably represents a previously unrecognized plasma protein. Monospecific antibodies raised to it were reactive with the purified urokinase-urokinase inhibitor complex from urine and with its nonreducible M, 50,000 component obtained after digestion with hydroxylamine, confirming its identity with the protein responsible for the binding of urokinase in urine.

The observation that the bulk of urokinase in urine is recovered as a complex with this urokinase inhibitor (7) is not inconsistent with the recovery of significant amounts of free inhibitor in the present purification method. Indeed, of the total urokinase-related antigen in urine (60 μg/liter), about 75% or 45 μg/liter occurs as urokinase-urokinase inhibitor complex. Thus, only 20% of the total urokinase inhibitor-related antigen present in urine (200 μg/liter) is complexed with urokinase, leaving the remaining 80% available for purification in the free form. The factors which regulate the formation of the urokinase-urokinase inhibitor complex may play a significant role in the regulation of urinary fibrinolysis.

The physiological importance of urinary fibrinolysis for the maintenance of both ductal patency and urinary tract hemo-
stasis, however, remains to be established. Interestingly, the protein was also detected in plasma, where it occurs at a concentration of approximately 2 \( \mu \)g/ml. In addition, with the use of an ELISA specific for urokinase-urokinase inhibitor complex, the time-dependent formation of this complex in plasma was demonstrated following the addition of urokinase in equimolar amounts to the inhibitor. Moreover, the second order rate constants in the presence and absence of heparin measured by the complex-specific ELISA were in good agreement with those obtained using the purified urokinase inhibitor from urine.

Previous studies have only reported very slow inhibition of high concentrations of urokinase by plasma (23, 24), although the major plasma proteins \( \alpha_1 \)-antitrypsin (23, 25), antithrombin III (23, 24, 26), \( \alpha_2 \)-macroglobulin (23, 24, 27, 28), and \( \alpha_2 \)-antiplasmin (29, 30) all slowly form complexes with urokinase. More recently it has been recognized that very low concentrations of urokinase are rapidly inhibited in plasma by a fast-acting inhibitor which neutralizes both urokinase and t-PA (31). Its estimated second order rate constant for urokinase inhibition is \( 10^7 \) M\(^{-1}\) s\(^{-1}\), which is at least \( 10^8 \) above that of the urokinase inhibitor described here and still \( 10^6 \) above that observed in the presence of heparin. However, physiological plasma concentrations of the fast-acting t-PA inhibitor are less than 5 ng/ml (32) or more than 400-fold less than that of the urokinase inhibitor. Thus, although the rate constant of the inhibition of urokinase by this novel inhibitor is small by comparison, it is not inconceivable that at its higher measured concentration it could play a role in the physiological inactivation of urokinase in plasma. The potential for such a role is supported by the observation of urokinase-urokinase inhibitor complex formation in plasma, although in a \(^{125}\)I-labeled fibrin plasma clot system we have thus far been unable to detect increased rates of lysis in plasma immunodepleted of this inhibitor. The potential role of the urokinase inhibitor would likely be most pronounced in the microvasculature where cell surface heparin-like substances would stimulate its urokinase inhibitory capacity. The availability of a purification procedure and a practical immunoassay for this protease inhibitor will facilitate further investigations of its biological function.

Acknowledgments—We acknowledge the skillful technical assistance of Eddy Demarsin and Lena Kieckens. We are also grateful for the kind assistance provided by Dr. D. Loskutoff (Scripps Institute, La Jolla, CA), Dr. J. Baker (University of Kansas, Lawrence, KA), Dr. E. Kruithof (Centre Hospitalier Universitaire du Vaudes, Lausanne, Switzerland), and Dr. J. Jacques (Behringwerke, Marburg/Lahn, West Germany) in the immunologic characterization studies.

\(^*\) D. C. Stump, M. Thienpont, and D. Collen, unpublished observations.

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