Isolation and Characterization of Urokinase from Human Plasma*

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The presence of activators of the fibrinolytic system in blood plasma has been assumed for a long time but never convincingly documented by the isolation of characterized and physiologically plausible enzymes. The low catalytic efficiency of previously identified plasma plasminogen activators, which has made their physiological significance uncertain, prompted us to search for other plasma enzymes, resembling especially the potent urinary activator, urokinase. We report here the detection of a urokinase-like activity in human plasma, and the isolation of the enzyme from whole plasma protein fractions. The purified enzyme is indistinguishable from the 53,000-dalton components of human urinary urokinase in the following respects: apparent $M$, on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, double immunodiffusion, amino acid analysis, two-dimensional tryptic peptide maps, catalytic efficiency with synthetic peptide substrates, and inhibitor spectrum. The results suggest that the enzyme is present in plasma in a latent form whose nature remains to be defined, and that the circulating concentration is at least 5 to 10 $\mu$g/liter, and sufficient to generate substantial levels of plasmin, particularly if activation were somehow confined by localization at specific sites.

Plasminogen activators catalyze the formation of plasmin by hydrolyzing a single peptide bond in the precursor, plasminogen, a proenzyme that is present at high concentrations in the blood and body fluids (1, 2). These enzymes are of interest because a growing body of evidence suggests that they participate in a wide variety of biological functions. Apart from its assumed fibrinolytic role in the regulation of blood clotting (3), plasmin has been implicated in complement activation (4), kinin formation (5), prohormone conversion (6), and the generation of localized extracellular proteolysis during tissue remodeling, cell migration, carcinogenesis, and neoplasia (7).

Plasminogen activators have usually been named according to the starting material from which they were isolated (e.g., urinary, or tissue, or cell culture plasminogen activators, etc.) (see Refs. 3, 8, and 9 for reviews), and the separate molecular identities of these enzymes has been in dispute (10). Immunological data, catalytic efficiency, and studies of specificity with small peptide substrates suggest that, in man, there are two distinct enzymes that are physiologically plausible plasminogen activators: the first is the urinary enzyme, urokinase (11), and the second is an activator isolated from uterine tissue (12), generally designated as tissue activator, which resembles an enzyme originally purified from pig heart (13). The enzymes variously isolated from cell culture (14), vascular endothelium (15), or from blood following venous occlusion (16) are probably all identical with one or another of the above.

The presence of a fibrinolytic pathway intrinsic to plasma, a plasminogen activator, and their identity, if they exist, are also unsettled questions. Coleman (17) reported that plasma kallikrein could activate plasminogen. Subsequently, Kaplan and Austen (18) described a partially purified plasminogen proactivator, ultimately shown to be inseparable from plasma kallikrein (19, 20). More recently, coagulation factor Xla has been proposed as a plasminogen activator (21), and plasminogen activation by factor Xla has also been observed (22). All three of these enzymes are of doubtful significance as physiologically relevant plasminogen activators, firstly, because catalytic activity (with plasminogen as substrate) is very sluggish, and, secondly, because genetically determined deficiency states give no indication of functional limitations that could reasonably be attributed to reductions in plasminogen activation or fibrinolysis.

In comparison with plasma kallikrein, human urinary urokinase is 10$^4$ to 10$^5$-fold more active as a plasminogen activator. The high catalytic efficiency suggested that if urokinase, or its proenzyme, circulated as normal constituents of plasma, trace concentrations might suffice to meet the functional needs for plasminogen activation. This consideration prompted us to undertake a search for urokinase in human plasma. In radioimmunoassays of plasma, Astedt (23) found occasional low levels of material that cross-reacted with rabbit antisera raised against urinary urokinase, and while he regarded these as being of questionable significance, his results added encouragement to the search for plasma urokinase. In what follows, we describe the isolation of a urokinase-like molecule from human plasma fractions, and the identification of the corresponding enzymatic activity in plasma.

**EXPERIMENTAL PROCEDURES**

**Materials**

CNBr-activated Sepharose 4B-CL, protein A-Sepharose, DEAE-Sephadec were obtained from Pharmacia, Inc., Piscataway, NJ; benzamidine hydrochloride, p-aminobenzamidine, 4-methylumbelliferyl-p-guanidinobenzoate, 4-methylumbelliferon, apetinin from Sigma; crude urokinase from Leo Pharmaceuticals (Ballerup, Denmark); BioRex 70 from Bio-Rad; fluorescamine from Hoffman-LaRoche; L-prolylglutamylglycyl-1-arginine-p-nitroanilide (S-2444), $\beta$-valyl-levucyl-tyrly-p-nitroanilide (S-2251) from Ortho Diagnostics; 125$I$-sodium iodide from Amerham Corp.; Spectravore 2 from VWR Scientific Inc., San Francisco. Aquacide II-A from Calbiochem-Behring; fresh human plasma from the New York Blood Bank; Cohn fractions III, IV, IV-1, and PPF plasma fractions were generously provided by Dr. P. Aiyappa, A. Guha, and E. Reich, unpublished results.

Duane Schroeder of Cutter Laboratories (Berkeley, CA); 6-aminoacryloxy-7-p-aminobenzamidine Sepharose was prepared as described elsewhere (24). Erythrinin inhibitor was supplied by Dr. Eugene B. Dowdle, University of Cape Town, Cape Town, South Africa. Trasylol was a gift of Dr. E. Truschtel, Bayer A. G., West Germany.

Methods

Electrophoretic Procedures and Zymography—Analytical SDS-PAGE was performed according to the method of Laemmli (25), using a slab gel plate (1 × 85 × 110 mm). The separating and stacking gels contained 10% and 4% acrylamide, respectively. Plasminogen activator activity of the protein bands separated on the gel was determined by the zymographic technique of Granelli-Piperno and Reich (26).

Purification of Urokinase—32 mg of commercially available urokinase (Leo Lot No. SE 766230 and Lot UDT A 93) containing about 200,000 Ploug units were dissolved in 2 ml of a buffer containing 0.1 M KPO4, pH 7.6, 0.4 mM NaCl, and applied to a benzamidine-Sepharose 4B column (1 × 15 cm). The column was washed with 100 ml of starting buffer, and urokinase was then eluted using either 2% acetic acid or 0.1 mM glycine/HCl, pH 2.2. A typical purification yielded 2.84 mg of urokinase (assuming Σ280nm = 13.4). The purified urokinase consisted of two major Cosmases blue-stainable bands (apparent M, = 52,000 and 58,000) on SDS-polyacrylamide gel electrophoresis. The nonreducing conditions. Numerous weakly stained minor bands were also visible in the regions corresponding to M, ~ 47,000 and 53,000 to 120,000. The corresponding proteins could also activate plasminogen when assayed by the zymographic technique of Piperno and Reich (26).

Immunization Procedures—Healthy New Zealand white rabbits were treated with an initial injection into the popliteal lymph nodes of 250 μg of purified urokinase (inactivated by iPr2-F as previously described (27)) suspended in 100 μl of complete Freund's adjuvant. Booster injections of 100 μg in 100 μl were administered 6, 7, and 8 weeks thereafter. Rabbits were bleed weekly beginning at 9 weeks and the titer of the anti-urokinase serum was determined by Ouchterlony double immunodiffusion analysis.

Purification of Immunoglobulin G—Sera from immunized or urokinase-immunized rabbits were adjusted to 40% saturation in (NH4)2SO4. The precipitate was dissolved in PBS supplemented with 0.4 M NaCl and dialyzed versus the same buffer. This solution containing 250 mg of protein in 5 ml was loaded onto a protein A-Sepharose column (4.7 ml bed volume). The column was washed with 5 column volumes of starting buffer and the IgG fraction was eluted with 0.1 M glycine/HCl buffer, pH 2.2, directly into collecting tubes that contained enough 1 M Tris/Cl, pH 9, to bring the eluate to pH 7.8. The yield was approximately 65 mg of protein. Alternatively, the immunoglobulin was purified by DEAE-Sepharose chromatography: the protein precipitated by (NH4)2SO4 was dialyzed against 0.1 M Tris/HCl, pH 8.8, 0.5% Triton X-100, and applied to a column of DEAE-Sepharose (2.5 × 32 cm). The column was developed with a gradient from 0.0 to 0.5 M NaCl, total volume 2 liters. Fractions containing immunoglobulin G were identified by SDS-PAGE and pooled.

Synthesis of Urokinase-Sepharose 4B-CL—0.5 mg of urokinase in 0.1 mM Na/MES, pH 6.5 (coupling buffer), was mixed with 2 ml of swollen cyanogen bromide-activated Sepharose 4B-CL previously equilibrated in coupling buffer and incubated in a water bath at 40°C for 30 min. SDS-PAGE was used to check that there was no detectable binding of urokinase-Sepharose 4B-CL to the gel. The column was developed with a gradient from 0.0 to 0.5 M NaCl, total volume 2 liters. Fractions containing immunoglobulin G were identified by SDS-PAGE and pooled.

Immunoaffinity Purification of Anti-Urokinase Ig—The IgG-containing fractions (20 mg/ml) obtained after protein A-Sepharose 4B affinity chromatography or DEAE-Sepharose ion exchange chromatography were pooled and passed through a urokinase-Sepharose 4B-Sepharose 4B-CL (1 × 2 cm bed volume) equilibrated in PBS supplemented with 0.4 M NaCl. After washing the column with 10 ml of starting buffer, anti-urokinase-Ig was eluted employing 0.1 M glycine/HCl, pH 2.2, and immediately neutralized with 1 M Tris/Cl. The protein recovery in the eluate was approximately 5% of the initial Ig.

Adsorption/Desorption of Plasma on BioRex 70—Fresh human plasma was centrifuged at 10,000 × g to sediment platelets and cellular debris and the supernatant was frozen and stored in 200-ml aliquots at −70°C. The frozen plasma samples (200 ml) were thawed in 37°C water bath and dialyzed against 2 liters of 10 mM benzamidine in double distilled water at 4°C for 3 h using Spectrapore No. 2 dialysis membrane. This preparation was cleared by centrifugation at 15,000 × g for 60 min and subsequently mixed with 0.4 M NaCl. The eluate was pooled (40 ml), adjusted to pH 5.2 by adding 4 ml of Na acetate, pH 4.5, and concentrated to 20 ml in a dialysis bag covered with Aquacide IIA.

Immunoaffinity Purification of Plasma Urokinase from Cohn Fraction IV I—Human plasma Cohn fraction IV-I (500 g) was mixed with 2300 ml of glass-distilled water and homogenized in a Waring blender for 3 min. The mixture was titrated to pH 2.0 by dropwise addition of 6 N HCl, stirred at room temperature for 30 min, neutralized to pH 7.4 by addition of 6 N NaOH, and stirred further at room temperature for 2 h. Benzamidine/HCl (11.8 g), NaCl (87.7 g), and aprotonin (20,700 kallikrein inhibitor units) were added. The total volume was adjusted to 3,000 ml and the solution centrifuged at 1,000 g for 15 min. The supernatant was then passed through a preimmune rabbit Ig-Sepharose column, the latter was washed with 200 ml of PBS supplemented with 1% Triton X-100, 0.4 M NaCl, and 0.1% benzamidine, pH 7.4 (buffer A), and then 50 ml of the same buffer containing no Triton X-100 (buffer B). Elution of urokinase was accomplished with 160 ml of 0.1 M glycine/HCl, pH 2.2. The preimmune rabbit Ig-Sepharose 4B column was washed in the same way, and no urokinase-related plasminogen activator was observed by zymography. Three preparations of the eluate of anti-urokinase-Ig-Sepharose 4B column were combined, adjusted to 0.1 M in benzamidine and neutralized to pH 7.4 by dropwise addition of 1 M Tris/Cl. The solution was subjected to a second cycle of affinity chromatography identical with the first except that the anti-urokinase-Ig-Sepharose 4B-CL column was smaller (1 × 1.1 cm), and was washed with smaller volumes, 30 ml of buffer A and 10 ml of buffer B. The urokinase was eluted in about 20 ml of 0.1 M glycine/HCl, pH 2.2, and concentrated to 1.5 ml in a dialysis bag covered with Aquacide II A.

Preparative-SDS-PAGE was used to check that the urokinase in the eluate had not been modified by affinity chromatography.

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The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MUGB, 4-methylumbelliferyl-p-guanidino benzoyl; iPr2-F, diisopropylfluorophosphosphate; PBS, phosphate-buffered saline; MES, 4-morpholinoethanesulfonic acid.
absorbance change at 405 nm were made 13 min and 15 min, respectively, after plasminogen addition. A linear relationship between the rate of absorbance change and amount of activator was obtained in the range 0.1 to 1 Ploug unit.

**Active Site Titration of Urinary Urokinase and Plasma Urokinase**—The molarity of enzymatically active urokinase in solution was determined in the conditions described previously for human urinary urokinase (11.8). Protein concentrations were determined by absorbance measurements at 280 nm using the specific absorbance of E_{280} of 10 unless more accurate figures were available from the literature, as in the case of plasminogen (17), urokinase purified by benzamidine-Sepharose 4B (13), and Ig (11.8). Proteins purified by preparative SDS-PAGE were measured by the fluorometric method of Elder et al. (30). Before protein determinations, the protein solutions were dialyzed extensively against 0.1% SDS. A 50-µl aliquot of the dialyzed sample was mixed with 0.25 ml of 0.1 M Na-borate, pH 9.2, and 0.025 mg/ml of acetonitrile, 1 ml was added and the sample was mixed on a vortex shaker. A standard curve was constructed using varying concentrations of bovine urokinase.

**Tryptic Peptide Mapping**—The radiiodination of proteins in polyacrylamide gel slices, tryptic digestion, and peptide mapping were carried out as described by Elder et al. (30).

**Amino Acid Analysis**—The protein solution (approximately 5 to 10 µg in 0.1% SDS) in Corning No. 5820 tube (10 x 75 mm) was adjusted to 0.2 M in trichloroacetic acid by adding 100% (w/v) trichloroacetic acid and incubated on ice overnight. The precipitate was recovered by centrifugation at 12,000 X g for 1 h. The precipitate and the wall of the tube were washed twice with 0.1 M Na-borate, pH 9.2, and 0.025 mg/ml of acetonitrile, and the samples were hydrolyzed at 110 °C for 24 to 72 h. The amino acid composition was determined with a Durrum Spinco D-500 amino acid analyzer according to the method of Moore and Stein (31). Cysteine was determined after performic acid oxidation according to the method of Hirs (32).

**Immunodiffusion Analysis**—Double immunodiffusion analysis according to the method of Ouchterlony and Nilsson (33) was carried out in 1% agar gels in 0.1 M sodium barbital, 1 Triton X-100, 50 mM benzamidine. The samples obtained from preparative SDS-PAGE were adjusted to 2% Triton X-100, then added to the punched wells and allowed to diffuse overnight at 37 °C.

**Radioimmunoassay for Urokinase**—A competitive radioimmunoassay for urokinase was developed by the following procedure. Commercial urokinase was purified by p-aminobenzamidine-Sepharose chromatography as described above, catalytically inactivated by treatment with P_{1-20}P (104) and then iodinated with 125I using a modification of the chloramine-T method (34). To free 125I-urokinase from material that had lost immunoreactivity during iodination the product was purified by immunoaffiniity chromatography on a column of urokinase-Sepharose (29). Antibodies were raised against highly purified urokinase as described above, goat anti-rabbit serum was obtained from Behring Calbiotech, and the respective IgG fractions were isolated by protein A-Sepharose chromatography. The tracer antigen (125I-P_{1-20}P-urokinase) was stored in PBS supplemented with bovine serum albumin (0.1 mg/ml) and stored at 0 °C. The binding capacity of the tracer mixture was 0.4 µCi/ml. The binding capacity of the IgG fraction was estimated by determining equivalence points with defined amounts of antigen using the Ouchterlony double immunodiffusion technique: for anti-urokinase IgG, it was found to be 150 µg/ml, and for goat anti-rabbit IgG, 250 µg/ml. To 500 µl of freshly isolated plasma (obtained from the New York Blood Center) were added 50 µl of tracer antigen and 100 µl of 1:1000 dilution of anti-urokinase IgG, and the samples were kept on ice overnight. Then, 10 µl of nonimmune rabbit IgG (170 µg/ml) were added as a carrier, and precipitation was achieved by addition of 10 µl of goat anti-rabbit IgG and incubation at room temperature for 4 h. The precipitates were recovered by centrifugation and the radioactivity determined in a Packard y scintillation spectrometer. The radioactivity of the supernatants was determined the same way. To define a standard curve dilutions of urokinase in the range 5 to 500 µg/ml were prepared and treated in the same way. The ratios of bound to total antigen (B/T) were calculated and transformed to their logit functions according to the method of Rodbard et al. (35) and logit BT values were plotted as a function of the logarithm of urokinase concentration (log c). The concentrations of urokinase in plasma were then derived from the position of the respective logit values on the standard curve. All dilutions were made with PBS containing 0.1% of bovine serum albumin.

**RESULTS**

**Presence of Urokinase-like Activity in Plasma**—Analysis of fresh plasma by a zymographic gel procedure (26), for the presence of plasminogen activators shows two bands of activity; these are entirely accounted for by prekallikrein and factor XII, and no activity is seen near the molecular weight regions, 53,000 or 33,000, which are characteristic of urinary urokinase. A similar analysis of plasma aged for 3 to 5 days in the cold showed a much richer spectrum of urokinase-dependent fibrinolytic bands corresponding especially to molecular weights above 100,000. When such aged samples were incubated for long periods (>24 h) to permit the development of activity bands, a weak zone of lysis appeared at a position corresponding to the migration of the 53,000 component of urinary urokinase.

Stronger evidence for the presence of a urokinase-like molecule was obtained by passing fresh citrated or heparinized plasma over a column of washed glass beads (CPG-10, Electro-Nucleonics Inc.). After washing extensively with PBS, the column eluate contained plasminogen-dependent fibrinolytic activity, and isolated by affinity chromatography on columns of purified antigen. The hydrolysis of S-2444 was fully resistant to 10^{-5} M soya bean trypsin inhibitor, a characteristic property of urokinase. 2) When analyzed by the zymographic gel procedure (26), the KSCN fraction contained a plasminogen activator whose electrophoretic mobility was identical with the 53,000 component of urinary urokinase. While these observations suggested that plasma contained either a urokinase-like molecule or its precursor, the concentration in plasma was too low, and the adsorptive capacity of glass bead columns was too limited, to permit the isolation of useful amounts by this procedure.

Since the "contact activation" and adsorptive properties of glass are considered to be less than a large part from the surface distribution of negative charge, we explored the use of the cation exchanger BioRex 70 for fractionating fresh plasma with the aim searching for urokinase-like enzymes. When fresh human plasma was adsorbed to BioRex 70 and eluted in the presence of benzamidine (as described under "Methods") the eluate contained plasminogen-dependent fibrinolytic activity in the 80,000 to 90,000 region that was visualized in the fibrin-agar overlay (Fig. 1, slot 1); no fibrinolytic activity was present in the unadsorbed fractions. In work to be described elsewhere it has been shown that the 80,000 to 90,000 activity is due largely, and perhaps entirely to kallikrein, factor XIIa, or a combination of the two. If this eluate was dialyzed to remove benzamidine, a new plasminogen-dependent fibrinolytic zone appeared in the 53,000 region (slot 2). Further development of activity and the appearance of lytic zones at lower molecular weight were achieved by incubating the di-
Fractions after chromatography on BioRex 70: detection by zymography after SDS-PAGE. For experimental details, see "Methods." Slot 1, BioRex 70 eluate prior to dialysis: activity present in the 80,000 to 90,000 region, due to factor XII and/or prekallikrein. Slot 2, BioRex 70 eluate dialyzed overnight in the cold against 0.02 M NaPO₄, buffer, pH 7.4, containing 5 mM EDTA: a prominent lytic zone has appeared at 53,000. Slot 3, the dialyzed eluate incubated at 37 °C for 1.5 h. Slot 4, the dialyzed eluate incubated at 37 °C for 8.5 h: the zones corresponding to factor XII and/or prekallikrein have disappeared, and a new zone at ~47,000 has appeared. Slot 5, the unadsorbed fraction obtained by passing the dialyzed BioRex 70 eluate (without incubation at 37 °C) through affinity purified anti-uropinase-Ig-Sepharose 4B column (1.1 x 2 cm) in the absence of benzamidine: the factor XII-prekallikrein has not adsorbed to the column, but the 53,000 zone has. Slot 6, the eluate obtained by eluting the anti-uropinase-Ig-Sepharose 4B (as described in slot 5) with 0.1 M glycine/HCl, pH 2.2: no urokinase-like activity is recovered. Slot 7, the eluate obtained by adsorbing the BioRex 70 eluate to anti-uropinase-Ig-Sepharose 4B in the presence of 0.1 mM benzamidine and eluting with 0.1 M glycine/HCl, pH 2.2: a 53,000 urokinase-like activity and a 100,000 lytic factor were recovered.

Isolation of Urokinase from Plasma Fractions—The procedure outlined above was reproducible and yielded quantities of enzyme sufficient for enzymological work but not for tests of purity or detailed characterization. The relative instability of the enzyme during isolation limited the application of BioRex chromatography on a large scale, an option also discouraged by the cost of fresh plasma. In searching for a partially enriched starting material, we assayed the standard Cohn fractions by the zymographic procedure (26) and identified the enzyme in fractions III, IV, and IV-1, of which the last proved to be the most convenient starting material for processing on a larger scale.

Isolation of Urokinase-like Activity from Cohn Fraction IV-1—Cohn fraction IV-1 was dissolved and acidified to pH 2.0 to inactivate residual protease inhibitors and then neutralized before application to immunoaffinity columns. The resolving power of these columns was influenced by several factors. 1) The presence of benzamidine (10 to 100 mM) throughout the procedure was essential for recovery of any activity, presumably because it protected the enzyme against degradation by other contaminating proteases. 2) Columns based on purified but otherwise unfractionated total Ig did not give satisfactory results: both their effective capacity, and the degree of purification of urokinase-like material were unacceptably low. 3) Columns based on affinity-purified, specific anti-uropinase Ig proved effective. Even so, the Cohn fraction IV-1 extracts required two cycles of immunoaffinity chromatography before achieving a degree of enrichment that permitted the isolation of highly purified plasma urokinase.

Under these conditions, optimal results were obtained only when the amounts of successfully bound protein were reduced, firstly by washing the column with buffers containing Triton X-100 (1%) before elution of enzymes and, secondly, by passing the extracts over a Sepharose column to which purified, rabbit preimmune IgG had been bound.

As seen from the specific activity data in Table I, the enzyme recovered after two cycles of immunoaffinity chromatography was quite impure, and was estimated to account for only a minor fraction of the total protein. Zymography of SDS-polyacrylamide gels showed plasminogen-dependent fibrinolytic activity in two regions corresponding approximately to 53,000 and 100,000, respectively (Fig. 2A). When the electrophoretic gels were stained with Coomassie blue, a distinct band corresponding to 53,000 was observed; it was clearly separated from the bulk of contaminating proteins which were concentrated predominantly in the zones of high molecular weight, suggesting that this urokinase-like component might be isolated by preparative SDS-PAGE, a procedure in which the enzymatic activity of urokinase survives essentially intact. To test for the retention of their characteristic electrophoretic mobility after purification, each of the fibrinolytic zones (a, b) and other nonlytic zones (c, d, e) were excised from lanes parallel to that shown in Fig. 2A. These were sliced, extracted

![Fig. 1. The presence of urokinase-like activity in plasma fractions after chromatography on BioRex 70: detection by zymography after SDS-PAGE.](http://www.jbc.org/)

![Fig. 2. SDS-PAGE and zymography of the immunoaffinity purified activator from Cohn fraction IV-1. A, the zymography.](http://www.jbc.org/)
by incubation at 37 °C overnight in 1 ml of NH₄HCO₃ (50 mM), and the solutions were lyophilized and then reanalyzed by SDS-PAGE and zymography with fibrin-agar overlays. The 53,000 lytic species had retained its mobility; it was not converted to any other detectable components. In contrast, the 100,000 band had disappeared completely and been replaced by a component with mobility inseparable from 53,000 urokinase, suggesting that the larger species, whatever its nature, might be a precursor to 53,000 urokinase.

The material recovered after two cycles of affinity chromatography was concentrated and subjected to preparative SDS-PAGE. Fractions of 1 ml were collected and an aliquot of each was analyzed by zymography for the presence of fibrinolytically active material migrating at 53,000. The active fractions containing 53,000 enzyme were pooled and assayed. A summary of the purification of Cohn fraction IV-1 enzyme is given in Table I: 47 μg of apparently homogeneous urokinase were recovered from a total of 3 kg wet weight of Cohn fraction IV-1. Because no quantitative assay of urokinase-like activity could be performed on the crude starting material, we do not have an exact estimate of the overall yield or degree of purification. The specific enzymatic activity of Cohn fraction urokinase was first determined by direct hydrolysis of the synthetic substrate S-2444. This procedure gave a value of 28,000 Ploug units/mg of protein (Table I), which is considerably below that previously reported for 53,000 urinary urokinase (12). However, when this value was normalized to the concentration of active enzyme measured by active site titration, the specific activity was identical with that of 53,000 urinary urokinase (Tables I and II). Thus, a large fraction of Cohn fraction enzyme consisted of enzymatically inactive protein. The inactive protein is almost certainly urokinase that was denatured and inactivated during immunoaffinity chromatography, rather than contaminating unrelated protein: this was demonstrated by subjecting highly purified 53,000 urinary urokinase to chromatography on the same immunoaffinity columns used for isolating Cohn fraction enzyme, the specific activity of the urinary enzyme recovered after elution was reduced to 30,000 Ploug units/mg of protein, but was unchanged after correction by active site titration. Evidently, a large fraction of adsorbed urokinase is irreversibly denatured during adsorption and elution from the anti-urokinase column. The electrophoretic mobilities of purified Cohn fraction enzyme, 53,000 and 33,000 urinary urokinase are compared in Fig. 3. The apparent molecular weights of Cohn fraction and 53,000 urinary urokinase are identical.

Comparison of Cohn Fraction Urokinase-like Enzyme and Urinary Urokinase—The parameters used for this comparison were catalytic activity with both small substrates and plasminogen, susceptibility to several macromolecular inhibitors, immunological specificity, electrophoretic mobility, amino acid composition, and two-dimensional maps of iodinated peptides resulting from tryptic digestion of the labeled proteins.

Amidolytic Activity, Plasminogen Activation, and Effect of Some Macromolecular Inhibitors—The Cohn fraction urokinase isolated after preparative SDS-PAGE retained full activity for ~3 weeks when stored in 0.1% SDS at 4 °C and slowly lost activity during a 6-month period. This concentration of SDS blocks all enzymatic functions, but activity was fully restored if SDS was removed from the protein by sequestration in micelles of nonionic detergents. We found that a final concentration of 0.5% Triton X-100 fully reactivated both urinary and Cohn fraction urokinase, and the two enzymes were accordingly compared under these conditions. The exact molarity of active enzyme in each case was determined by active site titration using hydrolysis of MUGB (29), and the amidolytic activity was then established by measuring the rates of hydrolysis of the urokinase substrate S-2444. When normalized for differences in molarity measured by active site titration the results (Table II) showed that, within the limits of experimental error, Cohn fraction and urinary urokinase were identical in amidolytic activity, both being less active, on a weight basis, than the 33,000 species urinary urokinase. The values observed here for urinary urokinase were very close to those reported by others (11).

Plasminogen activation by these enzymes was assayed by a method in which the product plasmin, was measured by hydrolysis of the chromogenic substrate S-2251. The data (Table II) again show identical activity for Cohn fraction and 53,060 urinary urokinase. The 33,000 component of urinary urokinase was less efficient in plasminogen activation than the 53,000 enzymes, in contrast both to its superiority in amidolysis of the small substrate S-2444, and to the report of Barlow and White (11). The different assays used may account for this discrepancy.

Resistance to most macromolecular protease inhibitors is a striking characteristic of urinary urokinase and analogous plasminogen activators of murine origin (36). We compared the effects of four inhibitors on Cohn fraction and 53,000

**Table II**

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<tr>
<th>Apparent specific activity of urokinase and Cohn IV-1 activator</th>
<th>Apparent specific activity*</th>
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<tr>
<td></td>
<td>S-2444 assay</td>
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<tr>
<td>Ploug units/mg</td>
<td></td>
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<tr>
<td>Urokinase, 33,000 dalton</td>
<td>207,000 ± 7,000</td>
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<tr>
<td>Urokinase, 53,000 dalton</td>
<td>119,000 ± 5,000</td>
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<tr>
<td>Cohn IV-1, 53,000 dalton</td>
<td>122,000 ± 3,600</td>
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*The activities were assayed by hydrolysis of S-2444 or S-2251 using urokinase (Leo) as reference standard enzyme. Concentration was measured by MUGB titration (as described under "Methods") using a 30-min incubation period at room temperature. Each specific activity value reflects the average of three independent determinations.
urinary urokinase, using the amidolytic assay with S-2444 as substrate: both enzymes behaved identically and were not inhibited by soybean trypsin inhibitor (20 μg/ml), lima bean trypsin inhibitor (30 μg/ml), Transylol (0.7 μg/ml), and Erythrina inhibitor (30 μg/ml).

**Immunological Properties**—When analyzed by Ouchterlony double immunodiffusion using either rabbit antisera or affinity purified antibodies, Cohn fraction urokinase gave a line of identity with 53,000 urinary urokinase and partial identity with 33,000 urinary urokinase (Fig. 4).

When incorporated into the fibrin agar overlay used for zymographic detection of fibrinolysis affinity purified anti-urokinase inhibited Cohn fraction enzyme as well as both components of urinary urokinase (Fig. 5).

**Amino Acid Analysis and Tryptic Peptide Mapping**—These two structural parameters provide further evidence for the identity of Cohn fraction and urinary urokinase. The data in Table III document the similarity between the two enzymes and are generally in accord with the previously reported values in the literature (11, 37). For several amino acids our values appear to differ significantly from those of previous workers. These differences may reflect slight variations in conditions of hydrolysis, and they do not obscure the overall similarity in amino acid composition between the plasma and urinary enzymes.

To obtain tryptic peptide maps, samples of Cohn fraction

![Fig. 5. Inhibition of the fibrinolytic activity of urinary urokinase and the activator from Cohn fraction IV-1 by affinity purified anti-urokinase Ig. SDS-PAGE and the zymography were performed as described under "Methods." A, a, A', and a' contain equal amounts of affinity-purified activator from Cohn IV-1. B, b, B', and b' contain equal amounts of benzamidine-Sepharose purified urokinase. The fibrin-agar for a, b, a', and b' contains 0.01 mg/ml of affinity-purified anti-urokinase-Ig; that for A, B, A', and B' does not. A, B, a, and b were incubated for 50 min. A', B', a', and b' were incubated for 120 min.](http://www.jbc.org/)

![Fig. 6. Tryptic peptide maps of the urinary urokinase and the activator from the Cohn fraction IV-1. A, 33,000 urinary urokinase; B, 53,000 urokinase; C, 53,000 enzyme from Cohn fraction IV-1. D, diagram of the major peptides in autoradiogram A to C. The shaded spots are missing in 33,000 urinary urokinase.](http://www.jbc.org/)

**Table III**

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<tr>
<th>Amino acid composition of urokinase and Cohn IV-1 activator</th>
<th>33,000 urokinase</th>
<th>53,000 urokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.0 ± 0.1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>19.1</td>
</tr>
<tr>
<td>Thr</td>
<td>20.0&lt;sup&gt;f&lt;/sup&gt;</td>
<td>20.0</td>
</tr>
<tr>
<td>Ser</td>
<td>20.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>20.2</td>
</tr>
<tr>
<td>Glu</td>
<td>33.9 ± 0.2</td>
<td>28.3</td>
</tr>
<tr>
<td>Pro</td>
<td>15.2 ± 0.1</td>
<td>16.4</td>
</tr>
<tr>
<td>Gly</td>
<td>29.4 ± 1.9</td>
<td>22.4</td>
</tr>
<tr>
<td>Ala</td>
<td>13.9 ± 0.5</td>
<td>10.1</td>
</tr>
<tr>
<td>Cys</td>
<td>15.1 ± 0.1</td>
<td>9.0</td>
</tr>
<tr>
<td>Val</td>
<td>11.5 ± 0.1</td>
<td>10.9</td>
</tr>
<tr>
<td>Met</td>
<td>4.3 ± 0.3</td>
<td>4.5</td>
</tr>
<tr>
<td>Ile</td>
<td>12.8 ± 0.1</td>
<td>15.6</td>
</tr>
<tr>
<td>Leu</td>
<td>24.4 ± 0.2</td>
<td>20.6</td>
</tr>
<tr>
<td>Tyr</td>
<td>11.8 ± 0.1</td>
<td>12.8</td>
</tr>
<tr>
<td>Phe</td>
<td>9.8 ± 0.1</td>
<td>9.3</td>
</tr>
<tr>
<td>His</td>
<td>9.0 ± 0.1</td>
<td>10.2</td>
</tr>
<tr>
<td>Lys</td>
<td>17.4 ± 0.3</td>
<td>17.3</td>
</tr>
<tr>
<td>Arg</td>
<td>15.5 ± 0.3</td>
<td>14.3</td>
</tr>
<tr>
<td>Try</td>
<td>N.D.</td>
<td>4.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> These values were obtained from 24-, 48-, and 72-h hydrolysates extrapolated to zero time unless otherwise indicated.

<sup>b</sup> Values from performic acid oxidation; relative to aspartic acid.

<sup>c</sup> Standard deviation.

<sup>d</sup> Values from 24-h hydrolysates.
and urinary urokinase were concentrated by SDS-PAGE, recovered in slices containing the respective bands, iodinated, digested with trypsin, and analyzed by two-dimensional electrophoresis and chromatography as described by Elder et al. (30). The pattern of iodinated tryptic peptides was then obtained by autoradiography. The autoradiograms in Fig. 6 show identical patterns for Cohn fraction and 53,000 urinary urokinase, and a largely, but not entirely similar iodinated peptide map for 33,000 urinary urokinase.

Radioimmunoassay of Urokinase in Human Plasma—Samples of fresh, pooled human plasma were obtained from a blood bank and assayed for urokinase-like antigens. The results of the radioimmunoassay are given in Fig. 7 and yield a mean value of 12 μg of urokinase/liter of plasma.

**FIG. 7.** Logit BT versus log c (ng/ml) plot of a radioimmunoassay for urokinase in human plasma. All concentrations were assayed in duplicate. The slope of the curve was obtained by linear regression analysis of the mean values of logit BT. Crosses indicate the standard values and open circles indicate the values obtained for pooled plasma samples.

**DISCUSSION**

Our data provide for the first time a substantial body of coherent evidence for the presence of a urokinase-like enzyme in human plasma. That being so, our results should be assessed in relation to three questions. 1) Are the enzymes we have isolated from Cohn fractions, and the activity generated from plasma, essentially identical with urinary urokinase? 2) If so, what is the form in which this urokinase, or a closely related molecule circulates in the plasma? 3) What might be the physiological relevance, if any, of the presence of urokinase, or some precursor form in plasma?

The answer to the first question can be obtained by considering the aggregate of our findings which show that urinary urokinase (53,000) and the Cohn fraction enzyme are indistinguishable by chromatographic, immunological, electrophoretic, catalytic, and structural criteria. Although we have not excluded minor differences in glycosylation or other analogous post-translational modifications, we conclude that these enzymes are otherwise essentially identical. Owing to the limiting amounts of material that could be purified directly from plasma we were unable to obtain as extensive a comparison between plasma and urinary urokinase: in this case, apparent identity was observed only in electrophoretic, chromatographic, and immunological behavior. However, since the Cohn fractions are derived entirely from plasma, there is no reason to doubt that the enzymes obtained from the two sources are both the same.

Although it seems safe to conclude that urokinase, or a molecule very closely related to it, is present in human plasma, we cannot yet identify the form in which it is circulating. Urokinase-like activity can be detected in zymographic gels after aging a plasma sample, and the enzyme shows latency during the early stages of fractionation, indicating that most of it is present in an inactive form. However, since we have not yet isolated an inactive form from plasma we do not know whether it is a proenzyme or exists as a complex with a specific inhibitor.

Whatever the precise nature of the inactive circulating form, it does seem likely that it is an intrinsic physiological constituent of plasma, and not simply a transient inhibitor complex targeted for removal and breakdown. Because numerous tissues secrete enzymes indistinguishable from urokinase to generate the localized proteolysis needed for a wide range of physiological processes, it might be considered that the enzyme we have isolated is merely released from a complex formed with one of the many protease inhibitors that are present at high concentration in blood and body fluids. This appears unlikely, because all of the known protease inhibitors, several of which bind rapidly to urokinase, form covalent complexes that are not dissociated under our conditions of enzyme purification. It also seems unlikely that the enzyme is liberated by proteolysis of such complexes, because proteolysis would be expected to yield a population of enzyme molecules that was either very heterogeneous in size, or contained molecular species larger than urokinase, or both. In fact, the enzyme obtained from Cohn fraction is observed in zymography of plasma is appreciably less heterogeneous on electrophoresis than the well characterized urinary urokinase.

We cannot as yet draw any conclusions concerning the physiological significance of plasma urokinase or prourokinase, if the latter exists. Our immunoassay data indicate that plasma contains at least 10 μg/liter of urokinase-reactive material, a figure that is consistent with the levels of enzyme extrapolated from the quantities observed in Cohn fraction. Given the catalytic efficiency of urokinase, even this low concentration could rapidly generate appreciable amounts of plasmin, especially if activation was localized, and (b) would yield a level of fibrinolytic activity significantly greater than that which could be generated by the much larger amounts of circulating prekallikrein, assuming that the latter is capable of functioning in blood as a plasminogen activator. These considerations suggest that urokinase, or its inactive precursor, should tentatively be considered as a plausible, physiological activator of fibrinolysis intrinsic to plasma, although other physiologically significant activators may also be present.

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