Biochimica et Biophysica Acta, 445 (1976) 763—773

BBA 67921

PURIFICATION AND CHARACTERIZATION OF TWO FORMS OF UROKINASE *

MERCEDES E. SOBERANO [a], ENG BEE ONG [a], ALAN J. JOHNSON [a], MILTON LEVY [a], and GUENTHER SCHOELLMANN [b]

[a] *Departments of Medicine and Pathology, New York University School of Medicine, New York, N.Y. 10016, and* [b] *Department of Biochemistry, Tulane University School of Medicine, New Orleans, La. (U.S.A.)*

(Received May 3rd, 1976)

Summary

Affinity chromatography on agmatine-Sepharose was used for the separation of two active forms of urokinase (EC 3.4.99.26) from partially purified human urinary urokinase. The approximate molecular weight of the heavier form was 47 000 and of the lighter 33 400. Both forms were homogeneous by sodium dodecyl sulfate gel electrophoresis and by ^{3}H-labeled diisopropylphosphorofluoridate and ^{14}C-labeled *p*-nitrophenyl-*p'*-guanidinobenzoate incorporation studies. The 33 400 mol. wt. form had a single chain, and the 47 000 mol. wt. form had two chains (33 100 and 18 600 mol. wt.) linked by disulfide bonds. The specific activity of the heavier form was 104 000 CTA units/mg protein, compared with 226 000 units/mg for the lighter form but the activities per mmol of active site (molar activities) of the two forms were almost identical ($9.6 \cdot 10^9$ and $10.2 \cdot 10^9$ CTA units/mmol). Isoelectric focusing on gels showed that the 47 000 material contained one major subform with a p*I* of 8.60 and a minor subform with a p*I* of 8.90, while the 33 400 material had three major subforms with p*I* values of 8.35, 8.60 and 8.70, respectively, and a minor subform with a p*I* of 8.05. ^{3}H-labeled diisopropylphosphorofluoridate incorporation studies revealed an active-site serine residue in the heavy chain.

Introduction

Over the past 15 years, a number of procedures have been reported for the purification of human urokinase (EC 3.4.99.26) the activator from human

* A preliminary report was presented at a meeting of the Federation of American Societies for Experimental Biology, 1975 (Fed. Proc. 34,860).
Abbreviation: iPr_2P-F, diisopropylphosphofluoridate.

urine capable of converting plasminogen to plasmin [1—5]. In 1965 Lesuk et al. [6] prepared crystalline urokinase, homogeneous by polyacrylamide gel electrophoresis and ultracentrifugation, which contained a single polypeptide chain of 54 000 mol. wt. and showed a pH-dependent association/dissociation reaction. White et al., however, isolated two types of urokinase (S_1 and S_2), of which S_1 was the more active, having a specific activity of 218 000 units/mg protein and a mol. wt of 31 600. S_2, with 54 700 mol. wt, did not appear to dissociate into subunits [7].

Partial purification of other plasminogen activators from tissues and from cell cultures has also been reported [8,9].

The present paper describes the purification of urokinase by affinity chromatography, characterizes the two active forms and demonstrates their probable relationship, and discusses the nature and stoichiometry of their inhibition by diisopropylphosphorofluoridate (iPr_2P-F) and *p*-nitrophenyl-*p'*-guanidinobenzoate hydrochloride. Inhibition of urokinase by iPr_2P-F and *p*-nitrophenylguanidinobenzoate has been studied [10] but the reaction of these reagents with the different forms of urokinase has not been previously reported.

Materials and Methods

Partially purified, human urokinase Lot 54 (92 000 CTA units/mg protein) was kindly supplied by Serono, Ltd. Plasminogen was prepared according to the procedure of Johnson et al. [11]. *p*-Nitrophenylguanidinobenzoate was obtained from Nutritional Biochemical. ^{3}H-labeled iPr_2P-F in propylene glycol containing 900 Ci/mol was ontained from New England Nuclear, and unlabeled iPr_2P-F was from Aldrich. The protein markers for the molecular weight determination were human fibrinogen (courtesy of J. Newman of this laboratory), chymotrypsinogen A (bovine pancreas) from Mann Research, and phosphorylase A from Sigma Chemical. All other reagents and chemicals were of the highest grade available.

Polyacrylamide-gel electrophoresis

Sodium dodecyl sulfate gel electrophoresis was performed according to the method of Weber and Osborn [12] in 7.0% gel, except that dithiothreitol was used for disulfide bond reduction and the samples were dissolved in phosphate buffer-sodium dodecyl sulfate in the presence of 8.5 M urea. Molecular weights were determined from a simultaneous run of a number of reference proteins which included phosphorylase A (94 000), fibrinogen (alpha chain approximately 70 900, beta chain 60 400, gamma chain 50 700) and chymotrypsinogen A (25 700).

Isoelectric focusing using 7.5% polyacrylamide gels was performed essentially by the method of Catsimpoolas [13]. The gels were stained with Coomassie Blue according to the procedure of Malik and Berrie [14].

Gels were scanned with a Gilford gel scanner and spectrophotometer; the peak areas were measured with a planimeter.

Gel slices (about 1.1 mm) were prepared with a simple gel feed previously described [15].

pH gradients on the gels were measured directly at 25°C with a micro-glass

electrode approximately 1.5 mm in diameter and a reference electrode (Desaga, Brinkman Instruments).

Assay methods

After electrophoresis, urokinase activity was determined on the entire gel by the fibrin plate method [16].

Plasminogen activator activity was also determined on eluates from the gel slices by the caseinolytic assay [17], the fluorometric fluorescamine assay [18], or a protamine method adapted to a Technicon Auto-Analyzer II [18]. Research Reference Standard Urokinase from the World Health Organization, containing 4800 CTA units per ampoule, was used as a standard.

Synthesis of Sepharose-ε-aminocaproyl-agmatine affinity column

Sepharose 4B was activated with CNBr (5 g/25 ml) at 4°C according to the procedure of March et al. [19]. A solution of 3.3 g ε-aminocaproic acid in 25 ml of 0.1 M phosphate adjusted to pH 9.2 was added to 25 ml of packed, activated Sepharose gel. The mixture was stirred in the cold for 48 h, filtered and washed with one liter of cold water. The product Sepharose-ε-aminocaproic acid was brought to a volume of 45 ml with distilled water. A solution of 40 mg (0.2 mmol) agmatine dihydrochloride in 5 ml water was added, the pH adjusted to 5.2, and 34 mg (0.18 mmol) of [3-(3-dimethylaminopropyl)] ethyl carbodiimide hydrochloride was added. The mixture was continuously adjusted to maintain a pH of 5.0 with 4 M NaOH. The pH stabilized after two hours, and the stirring was continued at room temperature overnight. The Sepharose-ε-aminocaproyl-agmatine was washed until the final rinse was negative by a modified Sakaguchi reaction, although the granules still gave a faint positive reaction. The resulting Sepharose-ε-aminocaproyl-agmatine material in 0.1 M phosphate buffer, pH 6.8, was found to bind 2.4 mg of trypsin per ml of packed gel.

Purification of urokinase by affinity chromatography

A 0.9 × 20 cm column of Sepharose-ε-aminocaproyl-agmatine was equilibrated with 0.01 M sodium phosphate buffer, pH 6.8, at 4°C, and 17.4 mg of partially purified commercial urokinase dissolved in the same buffer was applied to the top of the column. The column was first washed with 60 ml of the equilibrating buffer and then eluted with a step-wise gradient starting with 0.02 M sodium phosphate at pH 6.8, at a flow rate of 25 ml/h as shown in Fig. 1. All operations were conducted at 4°C. The column effluent was monitored for protein content by absorbance at 280 nm. Aliquots (10—20 μl) were taken from every other fraction, or from every fraction if making up a peak, and assayed for urokinase. Fractions were pooled as indicated in Fig. 1.

Samples (0.4 ml) of the pooled fractions were brought to pH 8.3 with *N*-ethylmorpholine for p-nitrophenylguanidinobenzoate titration. The remaining pooled material was reacted immediately with [^{3}H] iPr_2P-F as described below, and the dialyzed [^{3}H] iPr_2P-F urokinase was subjected to amino acid analyses as well as sodium dodecyl sulfate polyacrylamide gel electrophoresis.

p-Nitrophenylguanidinobenzoate titration

Titrations were carried out at 25°C essentially as described by Chase and Shaw [20] for trypsin.

The radioactive sample of ^{14}C-labeled *p*-nitrophenylguanidinobenzoate (2.13 Ci/mol) was synthesized according to the procedure of Chase and Shaw, utilizing p-amino[*carboxy*-^{14}C]benzoic acid as the starting material. Titration of urokinase with ^{14}C-labeled *p*-nitrophenylguanidinobenzoate was the same as with unlabeled material. The reaction mixture was dialyzed overnight against 4 M urea, pH 3.0, to remove excess labeled reagent. The dialyzed labeled urokinase was then subjected to dodecyl sulfate gel electrophoresis.

Reaction of urokinase with [^{3}H]iPr$_2$P-F

Assuming a mol. wt. of 33 000 for the starting material, titration with *p*-nitrophenylguanidinobenzoate indicated that approximately 33% of the commercial urokinase preparation was active. The urokinase sample was dissolved in 0.06 M *N*-ethylmorpholine, pH 7.5, to a concentration of 4.1 mg/ml. A two-fold molar excess of [^{3}H]iPr$_2$*P*-F ($8.04 \cdot 10^{-5}$ M) in isopropanol was added and the reaction mixture was incubated at room temperature. Duplicate 10-μl samples of the reaction mixture were assayed for activator activity by the automated assay at 5-min intervals for 30 min, and then at 15-min intervals for the next 30 min. After 60 min, a 5-fold molar excess of unlabeled iPr$_2$*P*-F ($2 \cdot 10^{-4}$ M) was added, the reaction was allowed to proceed for another 60 min, and the activator activity again determined. The reaction mixture was then dialyzed exhaustively at 4°C against 0.01 M phosphate buffer, pH 7.0, for 48 h. 50-μl aliquots were removed for titration with *p*-nitrophenylguanidinobenzoate at 0, 60 and 120-min incubation times.

The dialyzed [^{3}H]iPr$_2$*P*-F urokinase was examined by sodium dodecyl sulfate gel electrophoresis. The gels to be assayed for radioactivity were either sliced immediately after electrophoresis and eluted, or sliced after staining and destaining.

Pooled fractions from the affinity chromatography column corresponding to the 47 000 mol. wt and 33 400 mol. wt materials ($0.57 \cdot 10^{-5}$ M and $0.25 \cdot 10^{-5}$ M, based on protein concentration) were reacted in a 1 : 10 molar ratio with [^{3}H]iPr$_2$*P*-F. Activator activity was followed at room temperature at 15-min intervals for 90 min. A 50-fold molar excess of unlabeled iPr$_2$*P*-F was added and the reaction mixture was incubated for 30 min to ensure complete inactivation before the reaction mixture was exhaustively dialyzed against 0.01 M phosphate buffer, at pH 7.0. The dialyzed material was subjected to dodecyl sulfate gel electrophoresis.

Radioactivity measurements

Individual gel slices of radioactive samples after electrophoresis were eluted in scintillation vials containing 0.1% sodium dodecyl sulfate at 37°C for 18 h or more. These vials were then cooled, 10 ml of dioxan-based scintillant [21] was added, and they were assayed for radioactivity in a Model No. 3320 Packard Tri-Carb Scintillation Counter.

Protein determination

The protein concentration of solutions was estimated by ultraviolet absorption at 280 nm using Lot 54 urokinase as a standard with $E^{1\%}_{1\,cm} = 12.6$.

Protein samples were hydrolyzed for amino acid analysis at 110°C for 22 h

in 6 M HCl alone or with 0.1% mercaptoethanol and 0.2% phenol; the amino acid composition was determined on a Jeolco automatic amino acid analyzer attached to an Autolab AA integrator.

Results

Purification of urokinase by affinity chromatography

Rule and Lorand [22] described acetaminobenzene sulfonyl agmatine as an inhibitor of trypsin. The similar substrate specificity of urokinase and trypsin suggested the use of agmatine, coupled to a Sepharose matrix, as an affinity resin for the purification of urokinase. In our hands, recovery of urokinase from affinity columns ranged from 84% to 95%.

Partially purified starting material was resolved into four peaks of which only two showed urokinase activity (Fig. 1).

As measured by 280 nm absorbance, about 80% of the protein applied on the column was recovered in the eluates. Of the 17.44 mg applied, the first peak constituted 2.96 mg (17% of the total) of which pool A made up 55%. The fourth peak contained 4.73 mg (27% of total) of which pool D made up 31%. The inactive material made up a total of about 36% of the total amount of protein applied.

Results of dodecyl sulfate gel electrophoresis are shown in Fig. 2. The unreduced sample of pool A showed only one component, mol. wt. 33 400 ± 370 (N = 14) (Fig. 2a) and that of pool D, 47 000 mol. wt. ± 600 (N = 9) (Fig. 2c). Pools B and C contained inactive components of 18 500 and 74 000—96 000 mol. wt. We have designated the 33 400 mol. wt. and the 47 000 mol. wt. component isolated from the affinity chromatography column as AC-33 and AC-47. The molecular weight of AC-33 was not appreciably altered on reduction,

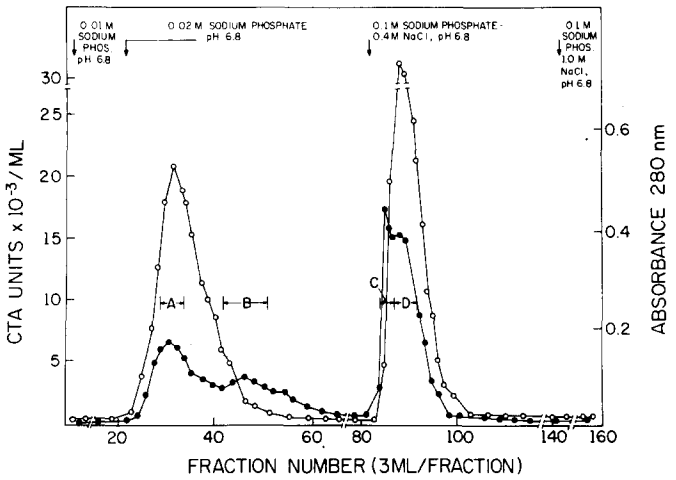

Fig. 1. Affinity chromatography of urokinase on Sepharose-ε-aminocaproyl-agmatine. 17.4 mg urokinase dissolved in 1.8 ml equilibrating buffer (0.01 M sodium phosphate, pH 6.8) was applied to a 0.9 × 20 cm column. Flow rate was 25 ml/h. Arrows indicate points of buffer change. Fractions were pooled as indicated. (○) Activator activity. (●) Absorbance at 280 nm.

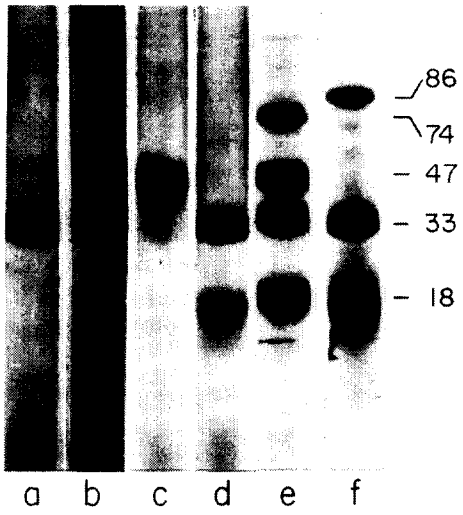

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of urokinase. Sample diluent consisted of 8.5 M urea, 1.0% sodium dodecyl sulfate and 0.01 M sodium phosphate buffer, pH 7.0. Reduction was achieved by dithiothreitol for 1 h at 37°C. AC-33 (a) unreduced and (b) reduced; AC-47 (c) unreduced and (d) reduced; starting material (e) unreduced and (f) reduced. Black line on (e) and (f) gels indicates extent of bromphenol blue migration, which was in a downward direction. The numbers on the right indicate the molecular weight in thousands.

33 100 ± 250 (N = 7) (Fig. 2b). On the other hand, bands corresponding to 33 100 ± 710 and 18 600 ± 670 (N = 7) mol. wt. were seen on reduction of AC-47 (Fig. 2d). Pool A had a specific activity of 226 000 CTA units/mg protein by the protamine assay, compared to 104 000 units/mg for pool D.

The starting material (Fig. 2e,f) showed four major bands in 7 runs corresponding to approximate molecular weights of 73 600 ± 1030 (13.2%), 47 300 ± 860 (32.9%), 32 700 ± 360 (25.8%) and 17 300 ± 430 (28.1%) with the unreduced sample. The values in parentheses refer to the percentage of distribution of each component. Components of 94 000 to 114 000 mol. wt. appeared as minor bands (<5%). On reduction, bands corresponding to 86 700 ± 1202 (15.9%), 32 700 ± 889 (41.9%) and 18 600 ± 695 (42.2%) mol. wt. were obtained. The highest molecular weight in the reduced sample was 86 700 and in the unreduced sample, 73 600. The higher value noted on reduction might indicate an aggregation of the low molecular weight components though this would be unlikely in the presence of 8.5 M urea, or the estimated value for the unreduced sample may be low. Molecular weights estimated by dodecyl sulfate gel electrophoresis tend to be low when disulfide bonds in the protein are intact [23].

Reaction of urokinase with iPr_2P-F

The reaction of highly purified AC-47 and AC-33 with a 10-fold molar excess of [^{3}H]iPr_2P-F (see Methods) at pH 6.8 followed pseudo-first-order kinetics. The second-order rate constants were then calculated by dividing the apparent first-order rate constants by the initial concentration of iPr_2P-F used. These calculations gave a k_2 value equal to 400 $M^{-1} \cdot min^{-1}$ for AC-47 and 770 $M^{-1} \cdot min^{-1}$ for AC-33.

When the starting material was incubated with iPr_2P-F (2 moles iPr_2P-F per mole active enzyme) in 0.06 M *N*-ethylmorpholine, pH 7.5, at 24°C, the loss of activator activity, as shown by assay after reaction with iPr_2P-F, paralleled the loss of available active sites as shown by *p*-nitrophenylguanidinobenzoate titration. The reaction followed second-order kinetics. The rate constant k_2 at pH 7.5, when calculated from the slope of a second-order plot, was 398 $M^{-1} \cdot min^{-1}$, Stoichiometric studies showed that about 0.36 mol of [^{3}H]iPr_2P-F was incorporated per mole of the urokinase preparation, and the active site titration showed an active fraction of 0.33 mol per mole of urokinase.

Dodecyl sulfate gel electrophoresis of [^{3}H]iPr_2P-urokinase

Dodecyl sulfate gel electrophoresis was performed on dialyzed [^{3}H]iPr_2P-F-treated urokinase preparations. Radioactivity measurements of gel slices of the unreduced and reduced AC-33 showed that the tritium labeling coincided with 33 400 mol. wt. band of the stained gel. Radioactivity of unreduced AC-47 was located in the 47 000 mol. wt. stained band but on reduction only the heavy chain was labeled (Fig. 3, A—D). Similarly, when electrophoresis was performed on the combined samples of pools A and D of Fig. 1, two radioactive peaks were noted, corresponding to 47 000 and 33 400 mol. wt. On reduction, there was only one radioactive peak corresponding to 33 100 mol. wt.

The protein band pattern of the dialyzed [^{3}H]iPr_2P-F-treated starting material was similar to that of the unmodified urokinase for both the reduced and unreduced samples. However, radioactivity was found only in the 47 300 and 32 700 mol. wt. bands of the sliced gels in the unreduced samples, and only in the 32 700 mol. wt. band in the reduced sample (Fig. 3, E and F). None was detectable in the other two bands — 73 600 mol. wt. and 18 600 mol. wt.

p-Nitrophenylguanidinobenzoate as an active-site titrant for urokinase

Titration with p-nitrophenylguanidinobenzoate revealed that only 68% of AC-33 was active enzyme, and 52% of AC-47; these low values may indicate the extreme lability of the highly purified forms. They may also suggest the presence of inactive enzymes which have the same or similar binding properties as AC-33 and AC-47 in the commercial starting material but no catalytic activities. The molar activities based on protamine assays and active site titrations were calculated to be $10.2 \cdot 10^9$ CTA units/mmol active enzyme for AC-33 and $9.6 \cdot 10^9$ CTA units/mmol for AC-47.

Dodecyl sulfate gel electrophoresis analysis of the [^{14}C]guanidinobenzoyl-urokinase starting material showed only two radioactive peaks, one corresponding to the 47 300 and the other to the 32 700 mol. wt. bands of the stained gel.

Isoelectric focusing

When isoelectric focusing was performed on the [^{3}H]iPr_2P urokinase at a pH gradient of 6—10, the [^{3}H]iPr_2P-F-labeled AC-33 showed three major peaks with isoelectric points of 8.40, 8.60 and 8.70, and one minor peak at pH 8.05 (Fig. 4A). The labeled AC-47 showed only one peak at pH 8.60 and a shoulder in the region of pH 8.9 (Fig. 4B). The peak at pH 10.4 did not appear consistently in all experiments. This finding, plus the fact that it appeared beyond the

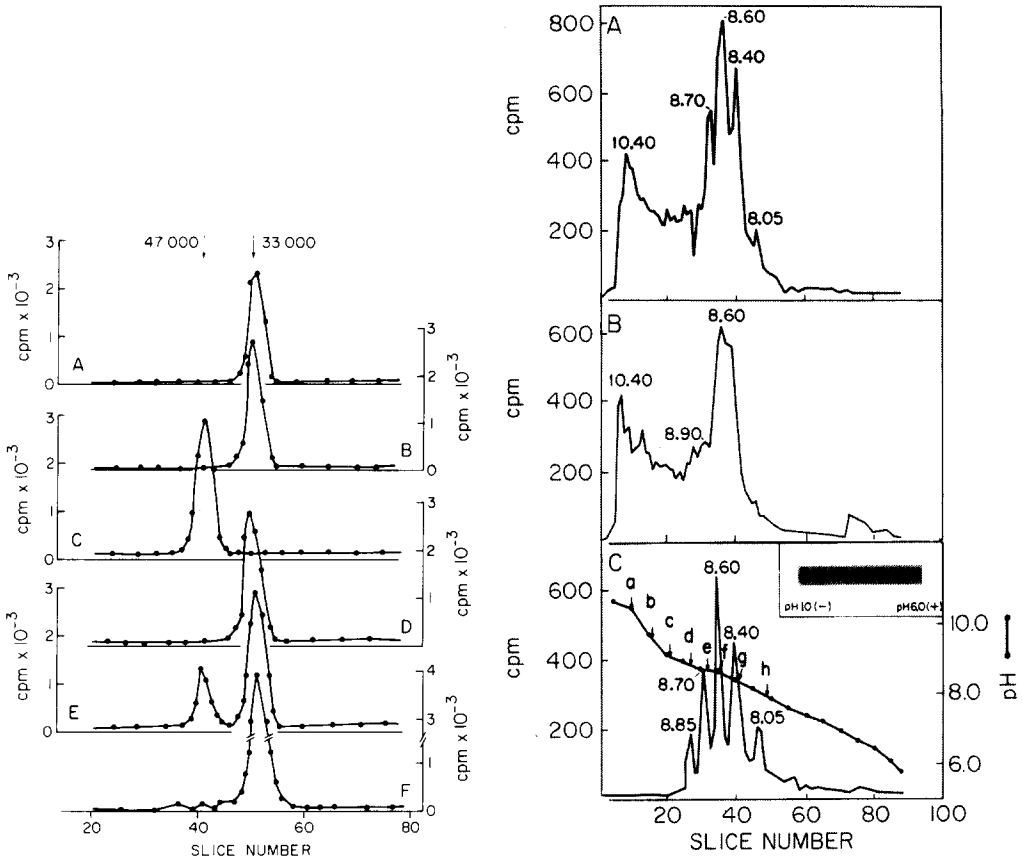

Fig. 3. Radioactivity patterns of dialyzed [^{3}H]iPr$_2$*P*-urokinase on sodium dodecyl sulfate gel electrophoresis. Each gel slice was eluted in 0.1% sodium dodecyl sulfate at 37°C overnight and assayed for radioactivity. AC-33 (A) unreduced and (B) reduced; AC-47 (C) unreduced and (D) reduced; starting material (E) unreduced and (F) reduced. Arrows indicate position of calculated molecular weights; migration was from cathode (left) to anode (right). No detectable radioactivity was observed in slices omitted from the figure.

Fig. 4. Radioactivity patterns of [^{3}H]iPr$_2$*P*-urokinase on isoelectric focusing in polyacrylamide gels. Focusing was performed in 7.5% gels over a pH range of 6—10. The pH gradient of replicate gels was determined directly on the gel layer with microelectrodes. (A) AC-33; (B) AC-47; (C) starting material. Inset shows gel stained with Coomassie Blue; isoelectric points of the stained bands are indicated by arrows on the pH curve, a to h, corresponding to pH values of 10.40, 9.40, 9.10, 8.85, 8.70, 8.60, 8.35 and 7.95, respectively. The isoelectric points of the radioactive peaks are also indicated on the peaks.

pH range of the ampholines, suggests that it is an artifact.

The [^{3}H]iPr$_2$*P*-F labeling of the starting material was found in bands with isoelectric points at pH 8.05, 8.40, 8.60, 8.70 and 8.85 (Fig. 4C). Those at pH 8.40, 8.60 and 8.70 constituted the major peaks.

Isoelectric focusing on gels of the starting material in the pH 6.0—10.0 range revealed, on staining, eight components with isoelectric points ranging from 8.0 to 10.4. The pattern of the bands is identical to that in the Fig. 4C inset. Caseinolytic and fluorometric assays indicated a high urokinase activity associated with the intensely stained bands in the pH 8.3 to 8.9 range. The fibrin plate assay also showed high activator activity in this pH range.

TABLE I

AMINO ACID COMPOSITION OF UROKINASE — AC-47 AND AC-33

	g%		Residues per 51 700 mol. wt. [b] AC-47	Residue per 33 400 mol. wt. AC-33
	AC-47	AC-33		
Lysine	8.47	7.27	35	19
Histidine	5.97	4.11	23	10
Arginine	7.24	7.79	25	15
Aspartic acid	9.58	8.10	43	24
Threonine	5.77 [a]	6.36 [a]	29 [a]	21 [a]
Serine	6.83 [a]	5.96 [a]	39 [a]	24 [a]
Glutamic acid	11.88	12.36	48	33
Proline	5.85	4.93	30	17
Glycine	5.60	5.07	45	27
Alanine	3.41	3.37	23	14
Half-cystine	3.97	2.98	20	9
Valine	4.23	4.13	22	11
Methionine	1.27	1.76	8 [c]	5 [c]
Isoleucine	3.99	5.68	18	15
Leucine	7.23	8.36	33	24
Tyrosine	5.64	5.83	19	12
Phenylalanine	3.92	3.91	14	9
Tryptophan	nd	nd	nd	nd
Total no. of residues			469	287

[a] Corrected for approximate decomposition of threonine (5%) and serine (10%).
[b] Mol. wt derived from the sum of the heavy and light chains (33 100 mol. wt. and 18 600 mol. wt.) by dodecyl sulfate gel electrophoresis of reduced AC-47.
[c] Determined as methionine sulfone after performic acid oxidation.

Amino acid analyses

The amino acid compositions of AC-47 and AC-33 (Table I) show no striking differences. The amino acid composition of AC-33 is similar to that of the B chains of the serine proteases, thrombin and plasmin, which also contain the active site peptides [24,25]. However, AC-33 has more threonine, serine and glutamic acid and less valine than the B chains of the other two enzymes.

Discussion

In this paper we have described a simple one-step isolation of two highly purified forms of urokinase from concentrates of commercial urinary urokinase by affinity chromatography on agmatine-Sepharose. The lower molecular weight form of the highly purified urokinase was found to be a single polypeptide chain of 33 400 mol. wt., with an active-site serine residue, as indicated by [^{3}H]iPr_2P-F incorporation studies. The higher molecular weight form had a molecular weight of 47 000, and consisted of two chains linked by disulfide bonds; the heavy chain contained the active-site serine, and this form appeared to be a precursor of the lower molecular weight form. In addition, our data suggesting that the higher molecular weight form is the physiologic form in

the urine agree with the findings of Lesuk et al. [6] but not with those of White et al. [7], who gave no information regarding the structure of the two separate (high and low molecular weight) fractions. Lesuk et al. [26] reported that their 54 000 molecular weight urokinase consisted of only a single polypeptide chain.

Kinetic analysis of the reaction of iPr_2P-F with AC-33 showed that the rate was about twice that of AC-47 alone, but the molar activity of AC-33 differs only slightly from that of AC-47. These findings suggest that AC-33 and AC-47 have a different rate of acylation, that the deacylation reaction is the rate limiting step, and that both forms have the same rates of deacylation. The value of 400 $M^{-1} \cdot min^{-1}$ for k_2 of the reaction of the starting material with iPr_2P-F is similar to the value of 300 $M^{-1} \cdot min^{-1}$ for trypsin [27], although Landmann and Markwardt [10] reported 1080 $M^{-1} \cdot min^{-1}$ for their urokinase, a discrepancy yet to be explained.

Isoelectric focusing of [^{3}H]iPr_2P-F-labeled starting material on polyacrylamide gels revealed radioactivity in 5 of the 8 stained bands, those at pH 8.05, 8.40, 8.60, 8.70 and 8.85. According to the isoelectric profiles, AC-33 appeared to be made up of components with isoelectric points of 8.05, 8.40, 8.60 and 8.70. AC-47 is made up principally of the component with an isoelectric point of 8.60 (a shoulder was evident at pH 8.4 and a minor component at pH 8.9). These findings seem to indicate that the 33 400 mol. wt. urokinase has the same major form as the 47 000 mol. wt. urokinase and some additional subforms. If these subforms arise from cleavage of a small peptide from the parent molecule, the difference in mol. wt. is too slight to be detectable on dodecyl sulfate gel electrophoresis. Alternatively, differences in the pls may be due to the presence of varying amounts of amino sugars or sialic acid in the molecules, as noted in the different subforms of plasminogen [28] or to deamidation of some glutaminyl or asparaginyl residues.

There was only a slight difference between the number of acidic and basic amino acids in AC-47; the same was true for AC-33 (Table I), the high p*I* values obtained for both suggest that the acidic amino acids are in their amide form, or amino sugars may be present.

Bergstrom [5] suggested that urokinase is likely to have a high p*I* value as it is avidly adsorbed by a strong cation exchanger at pH 10. We found much higher p*I* values than the 6.9 reported by Barlow and Lazer [29] for their urokinase. However, Walasek [30], also of Abbott Laboratories, recently reported the isolation of multi-molecular forms of urokinase with p*I* values from 7.5 to 9.8.

As might be expected, when experiments similar to the [^{3}H]iPr_2P-F incorporation studies were performed with ^{14}C-labeled *p*-nitrophenylguanidinobenzoate, only the 47 300 and 32 700 mol. wt. components of the starting urokinase preparation incorporated the [^{14}C]guanidinobenzoyl group.

Our incorporation studies with [^{3}H]iPr_2P-F and ^{14}C-labeled *p*-nitrophenylguanidinobenzoate strongly indicate an active site serine residue in AC-33 and the heavy chain of AC-47, and our other studies have identified histidine in the active site of urokinase [31], all observations which support the probability that urokinase is a classical serine protease. The similarity of the amino acid composition of AC-33 to the active-site containing B-chains of the serine proteases thrombin and plasmin also seems compatible with that conclusion.

Acknowledgments

We wish to thank Mrs. Elizabeth Dharmgrongartama for performing the amino acid analyses, and Mrs. Elma Wadsworth for editorial assistance. This work was supported in part by USPHS Grant HL-15596.

References

1 von Kaulla, K.N.(1954) J. Lab. Clin. Med. 44, 944—945
2 Ploug, J. and Kjeldgaard, N.O. (1957) Biochim. Biophys. Acta 24, 278—282
3 Celander, D.R. and Guest, M.M. (1960) Am. J. Cardiol. 6, 409—419
4 Sgouris, J.T., Storey, R.W., McCall, K.B. and Anderson, H.D. (1962) Vox Sang. 7, 739—749
5 Bergström, K. (1963) Ark, Kem. 21, 535—546
6 Lesuk, A., Terminiello, L. and Traver, J.H. (1965) Science 147, 880—882
7 White, W.F., Barlow, G.H. and Mozen, M. (1966) Biochemistry 5, 2160—2169
8 Kok, P. and Astrup, Y. (1969) Biochemistry 8, 79—86
9 Unkeless, J., Dano, K., Kellerman, G.M. and Reich, E. (1974) J. Biol. Chem. 249, 4295—4305
10 Landmann, H. and Markwardt, F. (1970) Experientia 26, 145—147
11 Johnson, A.J., Bergstrom, K. and Tse, A. (1967) Fed. Proc. 26, 2190
12 Weber, I. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
13 Catsimpoolas, N. (1968) Analyt. Biochem. 26, 480
14 Malik, N. and Berrie, A. (1972) Analyt. Biochem. 49, 173—176
15 Kamm, L. and Hurst, A. (1973) Analyt. Biochem. 53, 327—331
16 Vesterberg, O. and Eriksson, R. (1972) Biochim. Biophys. Acta 285, 393
17 Johnson, A.J., Kline, D.L. and Alkjaersig, N. (1969) Thromb. Diath. Haemorrh. 21, 259
18 Ong, E.B. and Johnson, A.J. (1973) Fed. Proc. 32, 413
19 March, S.C., Parikh, I. and Cuatrecasas, P. (1974) Analyt. Biochem. 60, 149—152
20 Chase, T. and Shaw, E. (1970) Methods Enzymol. 19, 20—27
21 Bray, G.A. (1970) in Determination of Radioactivity in Aqueous Samples (Bransome, E.D., ed.), p. 170, Grune & Stratton, New York
22 Rule, N.G. and Lorand, L. (1964) Biochim. Biophys. Acta 81, 130—135
23 Mann, K.G., Heldebrant, C.M. and Fass, D.N. (1971) J. Biol. Chem. 246, 5994—6001
24 Magnusson, S. and Hartley, B.S. (1972) in Atlas of Protein Sequence and Structure (Dayhoff, M.O. ed.), Vol. 5, D-109, The National Biomedical Research Foundation, Silver Spring, Md.
25 Groskopf, W.R., Hsieh, B., Summaria, L. and Robbins, K.C. (1969) J. Biol. Chem. 244, 359—365
26 Lesuk, A., Terminiello, L., Traver, J.H. and Groff, J.L. (1967) Fed. Proc. 26, 2194
27 Fahrney, D.E. and Gold, A.M. (1963) J. Am. Chem. Soc. 85, 997—1000
28 Siefring, G.E., Jr. and Castellino, F.J. (1974) J. Biol. Chem. 249, 7742—7746
29 Barlow, G. and Lazer, L. (1972) Thromb. Res. 1, 201—208
30 Walasek, O.F. (1975) Fed. Proc. 34, 2600
31 Ong, E.B., Johnson, A.J. and Schoellmann, G. (1976) Biochim. Biophys. Acta 429, 252—257