Purification of human urinary prokallikrein

Identification of the site of activation by the metalloproteinase thermolysin

Yasuharu TAKADA, Randal A. SKIDGEL and Ervin G. ERDÖS*

Departments of Pharmacology and Internal Medicine, The University of Texas Health Science Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235, U.S.A.

Human urinary active kallikrein and prokallikrein were separated on DEAE-cellulose and octyl-Sepharose columns and both purified to homogeneity by affinity chromatography, gel filtration and hydrophobic h.p.l.c. Prokallikrein was monitored during purification by trypsin activation followed by determination of both amidase and kininogenase activity. After trypsin activation, purified prokallikrein had a specific kininogenase activity of 39.4 μ g of bradykinin equivalent/min per mg and amidase activity of 16.5 μ mol/min per mg with D-Val-Leu-Arg-7-amino-4-trifluoromethylcoumarin. Purified active kallikrein had a specific activity of 47 μ g of bradykinin/min per mg. The molecular mass of prokallikrein was 48 kDa on electrophoresis and 53 kDa on gel filtration whereas active kallikrein gave values of 46 kDa and 53 kDa respectively. Antisera to active and prokallikrein were obtained. In double immunodiffusion and immunoelectrophoresis, antiserum to active kallikrein reacted with active and pro-kallikrein. Antiserum to prokallikrein contained antibodies to determinants not found in active kallikrein, presumably due to the presence of the activation peptide in the proenzyme. Human prokallikrein can be activated by thermolysin, trypsin and human plasma kallikrein. Activation of 50% of the prokallikrein (1.35 μ M) was achieved in 30 min with 25 nM-thermolysin, 78 nM-trypsin or 180 nM-human plasma kallikrein. Thus thermolysin was the most effective activator. Thermolysin activated prokallikrein by releasing active kallikrein with *N*-terminal Ile¹-Val². Thus human tissue (glandular) prokallikrein can be activated by two types of enzymes: serine proteinases, which cleave at the *C*-terminus of basic amino acids, and by a metalloproteinase that cleaves at the *N*-terminus of hydrophobic amino acids.

INTRODUCTION

The existence of a precursor of glandular or tissue kallikrein (EC 3.4.21.35) that is activated by trypsin has been noted in tissues such as pancreas (Werle & Urhahn, 1940), colon (Werle, 1960; Seki *et al.*, 1972), kidney (Nishimura & Erdős, 1980; Nishimura *et al.*, 1980*a,b*) or urine (Werle & Vogel, 1961). As kallikrein is easily inhibited by various proteins and peptides (Kraut *et al.*, 1930; Vogel, 1979), it was unknown initially whether an enhancement in kallikrein activity was due to the activation of a proenzyme or to the removal of an inhibitor. The properties of the human urinary prokallikrein and its mode of activation have not been characterized. Corthorn *et al.* (1979) partially purified a human urinary prokallikrein by monitoring its esterase activity. Spragg (1983) also reported briefly the purification of human prokallikrein, but without details. Silver *et al.* (1980) developed an r.i.a. for active urinary kallikrein, and Oza *et al.* (1981) assayed both prokallikrein and kallikrein in a single r.i.a. According to these authors, about one-half to two-thirds of the potential kallikrein activity in human urine can be attributed to the inactive, latent, kallikrein. Because we consider the enzymic-activation step of prokallikrein to be crucial for the understanding of the multiple functions of this enzyme in

kidney and elsewhere (Margolius, 1980, 1984; Nasjletti & Malik, 1981), we continued our studies on the conversion of renal and urinary prokallikrein into the active form (Nishimura *et al.*, 1980*a,b*; Nishimura & Erdős, 1980; Yamada & Erdős, 1982; Noda *et al.*, 1985). As described in this paper, we used human urine as a source of glandular (renal) kallikrein. We purified human urinary prokallikrein and active kallikrein to homogeneity from this source. We found that prokallikrein can be activated by enzymes that cleave either at the *C*-terminus of basic amino acids or at the *N*-terminus of hydrophobic amino acids to remove the activation propeptide sequence. In our experiments, the metalloproteinase thermolysin (EC 3.4.24.4) was the most efficient activator of prokallikrein, followed by trypsin (EC 3.4.21.4) and plasma kallikrein (EC 3.4.21.34).

EXPERIMENTAL

Materials

Thermolysin was purchased from Calbiochem-Behring Corp. La Jolla, CA, U.S.A. D-Val-Leu-Arg-F₃NMec and glutaryl-Ala-Ala-Phe-OMe-Nap were from Enzyme Systems Products, Livermore, CA, U.S.A. Trypsin was from bovine pancreas (type III, 12400 Bz-Arg-OEt units/mg), SBTI and dansyl chloride were from Sigma Chemical Co.,

Abbreviations used: r.i.a., radioimmunoassay; F₃NMec, 7-amino-4-trifluoromethylcoumarin; OMe-Nap, 4-methoxy-2-naphthylamine; Bz-Arg-OEt, benzoylarginine ethyl ester; dansyl or Dns, 5-dimethylaminonaphthalene-1-sulphonyl; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulphate.

* Present address and address for correspondence and reprint requests: Departments of Anesthesiology and Pharmacology, The University of Illinois College of Medicine at Chicago, 1740 West Taylor Street, Suite 3200 West, Box 6998, Chicago, IL 60680, U.S.A.

St. Louis, MO, U.S.A. DEAE-cellulose (DE 52) was from Whatman Ltd., Maidstone, Kent; U.K. *p*-Aminobenzamidine-Sepharose was from Pierce Chemical Co., Rockford, IL, U.S.A. Octyl-Sepharose CL-4B, Sephacryl S-200, Sephadex G-100, CNBr-activated Sepharose 4B and M_r markers for gel filtration were from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Affi-Gel Blue (agarose-bound Cibacron Blue F3GA) was from Bio-Rad, Richmond, CA, U.S.A. The TSK Phenyl-5PW column was from LKB-Produkter AB, Bromma, Sweden. Polyamide sheets and dansyl-amino acids were from BDH Chemicals, Poole, Dorset, U.K. Bradykinin was from Peninsula Laboratories, Belmont, CA, U.S.A. Antiserum to bradykinin was donated by Dr. O. A. Carretero, Henry Ford Hospital, Detroit, MI, U.S.A., and homogeneous human plasma kallikrein by Dr. A. P. Kaplan, State University of New York, Stony Brook, NY, U.S.A. The activity of the human plasma kallikrein preparation was $1400 \Delta A/\text{min}$ per mg of protein with the synthetic substrate Chromozym PK. Other reagents were laboratory grade or better.

Methods

Enzyme assays. Kallikrein activity was measured by a fluorimetric assay with D-Val-Leu-Arg-F₃NMeC as substrate (Noda *et al.*, 1985; Morita *et al.*, 1977) and by r.i.a. of bradykinin released from dog kininogen (Noda *et al.*, 1985). Amidase activity with D-Val-Leu-Arg-F₃NMeC is expressed as nmol of substrate hydrolysed/min (1 nmol/min = 1 munit). The fluorescence generated was measured in an Aminco-Bowman spectrofluorometer at 400 nm excitation and 505 nm emission wavelength. Kininogenase activity was determined by r.i.a. and is expressed as bradykinin equivalents released/min (Noda *et al.*, 1985).

Prokallikrein was routinely detected by activation with trypsin. Briefly, a 10–50 μl sample was incubated with 2.5 μg of trypsin in 0.1 M-Tris/HCl buffer, pH 8.0 (final volume 500 μl), for 30 min at 37 °C. After the reaction was stopped by the addition of 10 μl of SBTI (5 mg/ml), portions were assayed for kallikrein activity.

Purification of human urinary prokallikrein and active kallikrein. Human urine (101.5 litres) was collected from healthy volunteers into chilled bottles at 4 °C. A mixture of benzamidine, EDTA and NaN₃ in 0.1 M-Tris/HCl buffer (pH 8.0) was added periodically to yield final concentrations of 1 mM, 2 mM and 0.02% respectively. All subsequent procedures were carried out at 4 °C unless otherwise stated. Urine was filtered through filter paper (Whatman no. 1) and concentrated to 150 ml in an Amicon DC-2 hollow-fibre concentrator equipped with an H1P10 cartridge and in an Amicon ultrafiltration apparatus with a YM-10 membrane. The Tamm-Horsfall glycoprotein was then precipitated by adding NaCl to a concentration of 0.58 M (Corthorn *et al.*, 1979) and removed by centrifugation at 3000 *g* for 30 min. The supernatant solution was dialysed against 0.05 M-Tris/HCl buffer, pH 7.2, and then applied to a column (5 cm $\times$ 25 cm) of Affi-Gel Blue that had been equilibrated with the same buffer. The pass-through fractions were collected (1450 ml) and concentrated to 137 ml. The concentrate was dialysed overnight against 20 mM-sodium phosphate buffer, pH 6.0, and then applied to a DEAE-cellulose (DE 52) column (2.6 cm $\times$ 50 cm) equilibrated with the dialysis buffer. After washing with 1 litre

of the same buffer, the column was developed with a linear gradient of NaCl (0–0.5 M) in 20 mM-sodium phosphate buffer, pH 6.0. This technique separated prokallikrein and active kallikrein, which were eluted with 0.18 and 0.24 M-NaCl respectively.

Octyl-Sepharose CL-4B was packed into a column (2.6 cm $\times$ 27 cm) and equilibrated with 10 mM-sodium phosphate buffer, pH 6.8, containing 1 M-(NH₄)₂SO₄. The partially purified prokallikrein was first dialysed against the same buffer, then centrifuged at 8000 *g* for 20 min and the supernatant added to the column. After washing the column with 600 ml of the equilibration buffer, the protein was eluted with a decreasing gradient of (NH₄)₂SO₄ consisting of 150 ml of 1 M-(NH₄)₂SO₄ in 10 mM-sodium phosphate buffer, pH 6.8, and 300 ml of buffer. The tightly bound proteins were eluted with 50% (v/v) ethylene glycol in 10 mM-sodium phosphate buffer, pH 6.8. Active kallikrein, which was not completely removed on the DEAE-cellulose column, appeared in the pass-through fractions. Three prokallikrein peaks were observed: at conductivities of 36 and 21 mS [corresponding to 0.44 and 0.23 M-(NH₄)₂SO₄ respectively], and after elution with 50% ethylene glycol (Fig. 1 below). The prokallikrein fractions containing most of the activity (fractions 140–170, Fig. 1) were pooled and concentrated.

The concentrate (15 ml) was dialysed overnight against 50 mM-imidazole/HCl buffer, pH 7.0, and then applied to a column of *p*-aminobenzamidine-Sepharose (1.2 cm $\times$ 20 cm) equilibrated with the same buffer. After washing the column with the starting buffer, it was developed with a linear gradient of guanidinium chloride (0–0.35 M) in 50 mM-imidazole/HCl buffer, pH 7.0. Prokallikrein was eluted with 0.15 M-guanidine. Active kallikrein, which was generated during this step of purification, was eluted later with 0.25 M-guanidine. Fractions containing prokallikrein were pooled and concentrated to 3 ml by ultrafiltration, and then applied directly to a Sephacryl S-200 column (2.6 cm $\times$ 90 cm) equilibrated with 10 mM-sodium phosphate buffer, pH 7.2, containing 0.1 M-NaCl and 0.02% NaN₃. Fractions (3.3 ml each) were collected at a flow rate of 20 ml/h. Prokallikrein appeared in a single peak.

The final step in purification was carried out in a Waters gradient h.p.l.c. system (Skidgel *et al.*, 1984) on a TSK Phenyl-5PW column. The column was equilibrated with 0.1 M-Tris/HCl buffer, pH 7.4, containing 1.7 M-(NH₄)₂SO₄. Solid (NH₄)₂SO₄ was added to the concentrate from the Sephacryl S-200 column to a final concentration of 1.7 M, and a 400 μl portion (490 μg of protein) was injected. The proteins were eluted with a linear gradient of (NH₄)₂SO₄ decreasing from 1.7 M to 0 M in 0.1 M-Tris/HCl buffer, pH 7.4, over 30 min at a flow rate of 1 ml/min. At 280 nm, two peaks of protein were detected: a minor peak eluted at 23.0 min and the major protein peak, containing prokallikrein, at 28.6 min. Several injections were made and the peak eluted at 28.6 min was collected, concentrated to 1.7 ml and stored at –70 °C.

The active kallikrein, separated from prokallikrein on the DEAE-cellulose column, was further purified by *p*-aminobenzamidine affinity chromatography, gel filtration on Sephacryl S-200 and reverse-phase h.p.l.c. on a TSK Phenyl-5PW column under the same conditions as those described above. This preparation released 47 μg of bradykinin equivalent/min per mg and in SDS/poly-

acrylamide-gel electrophoresis gave a single band corresponding to a molecular mass of 46 kDa.

Preparation of thermolysin- and trypsin-Sepharose 4B. Thermolysin (71 mg, containing 50 mg of enzyme protein) and trypsin (50 mg) were covalently bound to 5 g of CNBr-activated Sepharose 4B according to the manufacturer's recommendations. Over 97% of each enzyme was coupled to Sepharose. The activities of thermolysin-Sepharose and trypsin-Sepharose were measured with glutaryl-Ala-Ala-Phe-OMe-Nap or D-Val-Leu-Arg-F₃NMeC substrates respectively. A 50 μ l portion of each sedimented gel was incubated with the corresponding substrate (0.1 mM) in 500 μ l of 0.1 M-Tris/HCl buffer, pH 8.0, containing 10 mM-CaCl₂ for 30 min on a turntable at room temperature. After centrifugation at 12000 *g* for 1 min, the fluorescence generated in the supernatant was measured in an Aminco-Bowman spectrofluorimeter at 340 nm excitation and 425 nm emission wavelength with glutaryl-Ala-Ala-Phe-OMe-Nap as substrate. With trypsin and the F₃NMeC substrate the two wavelengths were 400 and 505 nm. The activity of 50 μ l (sedimented gel) of thermolysin-Sepharose was equivalent to 1.2 μ g of free thermolysin, and that of 50 μ l of trypsin-Sepharose equivalent to 5 μ g of free trypsin.

Activation of purified prokallikrein. Purified prokallikrein (6.5 μ g; 1.35 μ M) was incubated with various concentrations of thermolysin (5–93 nM) trypsin (11–215 nM) homogeneous human plasma kallikrein (11–556 nM) or human urinary kallikrein (11–109 nM) in 100 μ l of 0.1 M-Tris HCl buffer, pH 8.0, for 30 and 60 min at 37 °C. The reaction was stopped by adding a mixture of phosphoramidon (1 μ M final concn) and SBTI (10 μ g) to inhibit completely thermolysin, trypsin or plasma kallikrein. In control studies the inhibitors were added before prokallikrein and they completely inhibited the activation. A 20 μ l portion of each sample was assayed for kininogenase activity by r.i.a. To determine the presence of traces of active kallikrein in the purified prokallikrein sample, an equal volume of the buffer was added to a 10-fold-concentrated sample (65 μ g) and assayed as described above. In an additional experiment, purified prokallikrein (2 μ g) was incubated with 100 μ l of sedimented gel of thermolysin- or trypsin-Sepharose in 500 μ l of 0.1 M-Tris/HCl buffer, pH 8.0, containing 10 mM-CaCl₂ for 10–240 min at room temperature. After centrifugation at 12000 *g* for 1 min, the activity in the supernatant was measured with D-Val-Leu-Arg-F₃NMeC as substrate.

Molecular-mass determination. The molecular mass of active and pro-kallikrein were estimated by gel filtration on a column of Sephadex G-100 (1.6 cm $\times$ 92 cm) equilibrated with 10 mM-sodium phosphate buffer, pH 7.2, containing 0.1 M-NaCl and calibrated with molecular-mass markers. The flow rate was maintained at 10 ml/h, and 2 ml fractions were collected. Kallikrein activity was measured with D-Val-Leu-Arg-F₃NMeC in each fraction before and after activation with trypsin or thermolysin (5 μ g/ml). For the determination of the molecular mass of thermolysin- and trypsin-activated kallikrein, purified prokallikrein (1 ml; 2 μ g) was incubated with 100 μ l of thermolysin- or trypsin-Sepharose for 1 h at room temperature. After centrifugation at 12000 *g*

for 2 min, the supernatant (1 ml) was applied to the column.

SDS/polyacrylamide-gel electrophoresis. SDS/polyacrylamide-gel electrophoresis was performed in 9% and 12% (w/v) slab gels at pH 8.6 by the method of Laemmli (1970). Samples were heated at 100 °C for 3 min in sample buffer with 1% 2-mercaptoethanol and then subjected to gel electrophoresis. Bovine serum albumin, ovalbumin, carbonic anhydrase, SBTI and lactalbumin (Sigma Chemical Co.) were used as protein standards. Protein was stained with 0.25% Coomassie Blue.

Determination of the N-terminus. The N-terminal amino acid residues of native and thermolysin-activated prokallikrein were determined by the dansylation technique (Gray, 1972; Hartley, 1970). Purified prokallikrein (200 μ g) was incubated with 100 μ l (sedimented gel) of thermolysin-Sepharose in 0.1 M-Tris HCl buffer, pH 8.0, containing 10 mM-CaCl₂ for 1 and 3 h at room temperature. After centrifugation at 12000 *g* for 2 min, the activity of the supernatant was measured with D-Val-Leu-Arg-F₃NMeC, and then applied to a column (0.75 cm $\times$ 30 cm) of TSK-G3000SW (Varian, Walnut Creek, CA, U.S.A.) in the h.p.l.c. system described above. Proteins were eluted with 0.07 M-sodium phosphate buffer, pH 7.2, containing 0.2 M-KCl at a flow rate of 0.3 ml/min. Essentially one protein peak was observed, which contained kallikrein activity. It was collected, dialysed against distilled water and then freeze-dried. Native prokallikrein (200 μ g) and prokallikrein (180 μ g) activated by thermolysin-Sepharose were heated at 100 °C for 2 min in 1% SDS and allowed to react with 25 mM-dansyl chloride in dimethylformamide for 2 h at room temperature. The dansylated protein was washed with acetone and then hydrolysed in 100 μ l of 6 M-HCl for 18 h at 105 °C. The hydrolysis products were identified by two-dimensional t.l.c. on a polyamide sheet (7.5 cm $\times$ 7.5 cm), dansyl-amino acids and dansyl-Ile-Val being used as standards. Three solvents were used for the chromatography: solvent 1, 1.5% (v/v) formic acid; solvent 2, benzene/acetic acid, 9:1, v/v; and solvent 3, ethyl acetate/methanol/acetic acid, 20:1:1, by vol.

Antibody preparation. Two New Zealand White rabbits were used for each immunization with active and pro-kallikrein. Purified prokallikrein (160 μ g) or active kallikrein (180 μ g) was emulsified in an equal volume of complete Freund's adjuvant (1 ml total) and injected subcutaneously at 2-week intervals a total of three times. A booster injection with the same amount of antigen was administered 4 weeks later in incomplete Freund's adjuvant. Serum was collected 2 weeks after the final injection and heated at 56 °C for 30 min.

Immunodiffusion and immunoelectrophoresis. Double immunodiffusion was performed as described by Ouchterlony & Nilsson (1978). Immunoelectrophoresis was done in 1.2% (w/v)-agarose gel in 50 mM-Tris/glycine buffer, pH 8.3, for 1 h at 2 mA/cm. The protein precipitate was stained with 0.25% Coomassie Blue.

Protein determination. Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Table 1. Purification of human urinary prokallikrein

Purification step	Volume (ml)	Protein (mg)	Activity* (units)		Sp. activity (units/mg)		Yield (%)		Purification (fold)	
			Kininogenase†	Amidase‡	Kininogenase	Amidase	Kininogenase	Amidase	Kininogenase	Amidase
Crude urine§	101 500	2460	529.5	182	0.215	0.074	100	100	1.0	1.0
Affi-Gel Blue	(150)									
DE52 ion-exchanger	1450	1093	504.2	169	0.461	0.154	95	93	2.1	2.1
Octyl-Sepharose	113	145.8	417.0	98	2.86	0.672	79	54	13.3	9.1
p-Amino-benzamidine-Sepharose	15	14.7	189.4	44.5	12.9	3.03	35.8	24.5	60.0	40.9
Sephacryl S-200	6	2.97	65.5	27.2	22.1	9.16	12.4	14.9	103	124
H.p.l.c.	1.8	2.46	59.7	26.5	24.3	10.8	11.3	14.6	113	148
	1.7	1.39	54.8	22.9	39.4	16.5	10.3	12.6	183	223

* Prokallikrein activity = (total activity after activation) – (active kallikrein activity).

† One unit of kininogenase activity equals 1 μ g of bradykinin released from kininogen-substrate/min at 37 °C.

‡ One unit of amidase activity equals 1 μ mol of D-Val-Leu-Arg-F₃NMec hydrolysed/min at 37 °C.

§ Tamm-Horsfall glycoprotein was removed; the value in parentheses is the volume after concentration.

|| This step separated prokallikrein from active kallikrein; the active-kallikrein peak contained 509 kininogenase units of active kallikrein and only 11.5 kininogenase units of prokallikrein; the prokallikrein peak contained only 16.9 kininogenase units of active kallikrein.

Fig. 1. Octyl-Sepharose column chromatography of human urinary prokallikrein

Elution was performed with a decreasing $(\text{NH}_4)_2\text{SO}_4$ gradient as described under 'Methods'. Only the major peak of prokallikrein, eluted in fractions 140–170, was purified further. Prokallikrein (●) was determined, after activation with trypsin, with D-Val-Leu-Arg-F₃NMec as substrate and active-kallikrein activity (○) was subtracted. . . . , protein; Δ, conductivity. The first arrow marks the start of the $(\text{NH}_4)_2\text{SO}_4$ gradient; the second arrow marks the start of the 50% ethylene glycol.

RESULTS

Purification of human urinary prokallikrein

The purification steps followed by r.i.a. of kinin released from kininogen are summarized in Table 1. The results obtained by measuring amidase activity with F₃NMec-labelled tripeptide substrate are also shown in Table 1 for comparison. The results with the two types of determinations were quite similar.

During the first step of purification on Affi-Gel Blue,

the urinary carboxypeptidase (Skidgel *et al.*, 1984) and albumin were retained, whereas prokallikrein appeared in pass-through fractions. Ion-exchange chromatography on a DEAE-cellulose column separated prokallikrein from active kallikrein, although the peaks were incompletely resolved. No other amidase activity was observed. Subsequently, prokallikrein was retained on the octyl-Sepharose column and separated from the remaining active kallikrein, which emerged in the pass-through fractions (Fig. 1). A small amount of prokallikrein was

Fig. 2. SDS/polyacrylamide-gel electrophoresis of purified human urinary prokallikrein

Left lane, protein molecular-mass markers; right lane, 25 μ g of purified prokallikrein. Protein was stained with 0.25% Coomassie Blue.

tightly bound to the column but was eluted with 50% ethylene glycol.

The final purification step, reverse-phase h.p.l.c. on a TSK Phenyl-5PW column, was employed to remove a small contaminant protein with a molecular mass of 32 kDa as established in SDS/polyacrylamide-gel electrophoresis. This column removed not only contaminating protein but also the yellow colour of the enzyme solution.

Prokallikrein in the crude urine concentrate was 49% of the total kininogenase activity and 54% of the total amidase activity. Prokallikrein was purified 223-fold when activity was measured with D-Val-Leu-Arg-F₃NMeC substrate and 183-fold with the r.i.a. The recovery of activity was 12.6% with fluorometric assay and 10.3% with kininogenase assay. Purified prokallikrein, after activation, had a specific activity of 16.5 μ mol/min per mg with D-Val-Leu-Arg-F₃NMeC as substrate and released 39.4 μ g of bradykinin equivalent/min per mg from dog kininogen. The preparation contained only traces of active kallikrein with a specific activity of 0.34 μ mol/min per mg with D-Val-Leu-Arg-F₃NMeC or 0.60 μ g of bradykinin equivalent/min per mg with dog kininogen. The purified enzyme was stable at -70°C in 0.1 M-Tris/HCl, pH 7.4, for 6 months.

Electrophoresis and immunodiffusion

The purified prokallikrein migrated as a single protein band in polyacrylamide-gel electrophoresis under denaturing and reducing conditions (Fig. 2). A broad band was obtained with 25 μ g, but it was observed even when a smaller amount of the enzyme was subjected to SDS/polyacrylamide-gel electrophoresis, indicating microheterogeneity of prokallikrein, possibly because of variation in carbohydrate content. The apparent molecular mass was 48 kDa in either 9%- or 12%-polyacrylamide gels in the presence of SDS.

Immunodiffusion experiments using antiserum to active kallikrein showed a single precipitin line against either active or pro-kallikrein, which fused completely (Fig. 3a). When antiserum to prokallikrein was placed in the centre well, precipitin lines formed with both active and pro-kallikrein. However, the prokallikrein precipitin

Fig. 3. Double immunodiffusion of purified active and pro-kallikreins against antiserum to active or pro-kallikrein

(a) The centre well contained 7 μ l of antiserum to active kallikrein; wells 1 and 3, 0.25 and 0.13 μ g of prokallikrein; wells 2 and 4, 0.28 and 0.14 μ g of kallikrein; well 5, 1.6 μ g of human plasma kallikrein. (b) The centre well contained 18 μ l of antiserum to prokallikrein; wells 6 and 8, 0.51 and 0.25 μ g of prokallikrein; wells 7 and 9, 0.56 and 0.28 μ g of active kallikrein; well 10, 1.6 μ g of human plasma kallikrein. The plates were stained with 0.25% Coomassie Blue.

line formed a spur and did not fuse completely with that of active kallikrein (Fig. 3b). Thus both active and pro-kallikrein completely cross-react with antibody against active kallikrein, but the antigenicity of prokallikrein is different in part from that of active kallikrein, as shown with antibody to prokallikrein. Some antigenic determinants, probably on the activation peptide, are lacking in the active kallikrein molecule as compared with prokallikrein. No cross-reactivity was observed with plasma kallikrein against antiserum to urinary active kallikrein or prokallikrein. Both active and pro-kallikrein gave a single arc of precipitation against antiserum to prokallikrein in immunoelectrophoresis (Fig. 4). A precipitin arc of active kallikrein appeared in a more anodic position than that of prokallikrein. No precipitin arc due to the presence of active kallikrein in the purified prokallikrein preparation was detected.

Fig. 4. Immunoelectrophoresis of human urinary active and pro-kallikrein

Purified active (0.22 μ g) and pro-kallikrein (0.2 μ g) were subjected to electrophoresis in a 1.2% agarose gel at pH 8.3 for 1 h at 2 mA/cm. Upper trough, anti-prokallikrein serum; lower trough, anti-(active kallikrein) serum. Abbreviation used: KK, kallikrein. Protein was stained with 0.25% Coomassie Blue.

Fig. 5. Activation of human urinary prokallikrein by thermolysin, trypsin and human plasma kallikrein

Purified prokallikrein (1.35 μ M) was incubated with thermolysin ($\square$), trypsin ($\triangle$) or plasma kallikrein ($\bullet$) for 30 min at 37 $^{\circ}$ C. Kallikrein activity was measured by r.i.a. of released kinin.

Activation by thermolysin, plasma kallikrein and trypsin

Fig. 5 shows the activation of homogeneous prokallikrein (1.35 μ M) by increasing concentrations of thermolysin, trypsin or plasma kallikrein. The residual, trace kininogenase activity of prokallikrein was 6.3 ng of bradykinin equivalent/min per ml before activation. The activity increased to a maximum of 260 ng with 47 nM-thermolysin, 256 ng with 215 nM-trypsin and 267 ng of bradykinin equivalent/min per ml with 556 nM-plasma kallikrein after 30 min. This optimum level of activity did not change after prolonged 60 min incubation. Thermolysin was the best activator, as the molar ratios of thermolysin (25 nM), trypsin (78 nM) and plasma kallikrein (180 nM) required for 50% of the maximum activation were 1:3:7.

To determine whether an autoactivation of prokallikrein would be possible, active human urinary kallikrein (11–109 nM) was tested as an activator, but it did not activate prokallikrein.

When purified kallikrein (2 μ g) was incubated with thermolysin- or trypsin-Sephadex (100 μ l of sedimented gel each) for 10–240 min at room temperature, the

activity with D-Val-Leu-Arg-F₃NMec substrate increased from 0.6 to about 27 nmol/min per ml within 10 min and stayed at this level during the remainder of the incubation. The maximal activation was equivalent to that obtained with 47 nM-free thermolysin or 215 nM-free trypsin.

K_m values with native, purified active kallikrein, and thermolysin- or trypsin-Sephadex-activated prokallikrein were determined from Lineweaver-Burk plots at eight different concentrations of D-Val-Leu-Arg-F₃NMec substrate ranging from 4 to 40 μ M in 0.1 M-Tris/HCl buffer, pH 8.0. The K_m values of the substrate with the three kallikreins were identical, 63 μ M, indicating that thermolysin and trypsin fully activated prokallikrein without further altering the catalytic site.

The apparent molecular mass of purified prokallikrein was 53 ± 2 kDa as determined by gel filtration on a Sephadex G-100 column. This value was higher than that obtained by SDS/polyacrylamide-gel electrophoresis (48000), possibly due to the glycoprotein nature of prokallikrein. The molecular masses of purified active kallikrein and thermolysin- or trypsin-Sephadex-treated prokallikrein were not significantly different from that of native prokallikrein (53 ± 2 kDa) in gel filtration.

N-Terminal amino acid

To determine which peptide bond is cleaved by thermolysin in the prokallikrein molecule, the N-terminus of prokallikrein before and after activation by thermolysin was examined. Alanine was the only N-terminal amino acid found with native prokallikrein, indicating homogeneity of the enzyme preparation. Isoleucine, Ile-Val and alanine were detected as the major spots and a faint valine spot was found after purified prokallikrein was activated with thermolysin-Sephadex for 1 h. After prolonged incubation for 3 h, the alanine spot could not be detected on a thin-layer plate, whereas the isoleucine and Ile-Val spots remained unchanged. This result indicates that thermolysin releases the N-terminal propeptide from prokallikrein and that the newly revealed N-terminal (Ile¹) of active kallikrein was linked to Val², in agreement with the reported N-terminal sequence of active human urinary kallikrein (Lottspeich *et al.*, 1979).

DISCUSSION

We report here the first complete purification of human urinary prokallikrein and its activation by serine proteinases and a metalloproteinase. Because of the possibility that other serine proteinases may also cleave synthetic short substrates, both amidase and kininogenase activities of kallikrein were assayed during purification.

Urinary kallikrein originates from the kidney (Margolius, 1984; Scicli *et al.*, 1978). Its molecular mass, inhibition pattern and substrate specificity are similar to, or identical with, those of the glandular or tissue kallikrein, present in many organs (Fiedler, 1979). The properties of this tissue kallikrein, however, are quite different from those of plasma kallikrein (Movat, 1979; Fig. 3).

Extensive studies on urinary kallikrein were prompted by observations of decreased excretion of kallikrein in some forms of hypertension (Elliot & Nuzum, 1934; Margolius, 1980) and of the effects of kinins on prostaglandin release (Nasjletti & Malik, 1981; Abe *et al.*, 1981), sodium excretion and water reabsorption (Margolius, 1984). Kinins filtered from blood by the glomerulus

are inactivated in the brush border (Ward *et al.*, 1975, 1976; Hall *et al.*, 1976). However, kinins are released by renal kallikrein, which enters the nephron in the distal tubule (Scicli *et al.*, 1978) or the circulation from the basolateral membrane of the tubular cells (Yamada *et al.*, 1981; Yamada & Erdős, 1982; Nolly & Lama, 1981; Vio *et al.*, 1981, 1983). Prokallikrein has been extracted from renal tissues (Nishimura & Erdős, 1980; Nishimura *et al.*, 1980a,b) and its release from kidney was noted (Nolly & Lama, 1981). Nishimura *et al.* (1983) partially purified a rat renal prokallikrein after using proteolytic inhibitors, and Takaoka *et al.* (1982) separated rat urinary prokallikrein from active kallikrein by ion-exchange chromatography.

Our purification, consisting of six steps, separated active and pro-kallikrein completely. The purified human urinary proenzyme appeared to be homogeneous. As observed with human urinary kallikrein, variation in carbohydrate content may cause a microheterogeneity in polyacrylamide-gel electrophoresis (Ikekita *et al.*, 1983; Morichi *et al.*, 1984). Antisera to active kallikrein did cross-react with prokallikrein and vice versa. However, antisera to prokallikrein contained antibodies to determinants (presumably of the propeptide) not found on active kallikrein.

Purified prokallikrein, active kallikrein and thermolysin- or trypsin-Sepharose-activated prokallikrein had similar molecular masses in gel filtration (53 ± 2 kDa). This was not unexpected, because in rat prokallikrein (Swift *et al.*, 1982), the activation peptide contains only 11 amino acids. Thus, if activation of human prokallikrein only changes the molecular mass by about 1 kDa, this would not be detected in gel filtration.

The *N*-terminal amino acid of prokallikrein is alanine as determined by the dansylation technique, but the *N*-terminal end could have been modified by aminopeptidases during processing in the kidney or after excretion into the urine. After activation by thermolysin, Dns-Ile, Dns-Ile-Val and Dns-Ala were found, in good agreement with the reported Ile¹-Val² at the *N*-terminus of active human urinary kallikrein (Lottspeich *et al.*, 1979). The trace amount of Dns-Ala found came from the *N*-terminal end of the remaining proenzyme, because it disappeared after prolonged incubation with thermolysin-Sepharose. The release of the *N*-terminal Ile-Val was expected, as this bond is resistant to acid hydrolysis (Gray, 1972). These data indicate that thermolysin cleaved the bond between the activation peptide and kallikrein in the prokallikrein molecule.

Thermolysin, after activating prokallikrein, did not cleave the released active kallikrein any further. When chymotrypsinogen is activated, its newly formed *N*-terminal end (Ile) is protected against further degradation by folding down into the molecule, where it forms an ionic bond with Asp-194 (Kraut, 1971). A similar mechanism may be involved in the activation of glandular kallikrein.

Prokallikrein was activated by serine proteinases as well. It is known that trypsin (Werle & Urhahn, 1940; Werle, 1960; Werle & Vogel, 1960, 1961; Corthorn *et al.*, 1979; Spragg, 1983), plasmin or pronase (Noda *et al.*, 1985) can activate human renal (Nishimura & Erdős, 1980) and urinary prokallikrein. Crude prokallikrein preparations may contain protein inhibitors (Kraut *et al.*, 1930; Vogel, 1979); thus an apparent increase in activity could be due to the destruction of an inhibitor as well as to the activation of the proenzyme.

In addition to the enzymes mentioned, human plasma kallikrein activates homogeneous urinary prokallikrein to the same extent as the other enzymes, although at higher concentrations. Trypsin-like serine proteinases cleave peptides and proteins at the carboxy end of basic amino acids. The nucleotide sequence of rat pancreatic preprokallikrein (Swift *et al.*, 1982) indicates that the propeptide is linked to active kallikrein through an Arg⁻¹-Val⁺¹ bond. Arginine is also the C-terminal amino acid of the activation peptide in prorenin (Imai *et al.*, 1983), which also can be activated, at least *in vitro*, by trypsin, kallikrein and plasmin (Sealey & Atlas, 1984; Schalekamp & Derkx, 1981). The same type of basic-to-hydrophobic-amino-acid link was also found, for example, in the precursors of trypsin (MacDonald *et al.*, 1982a), epidermal-growth-factor-binding protein (Lundgren *et al.*, 1984), parathyroid hormone (Habener *et al.*, 1984), chymotrypsinogen and proelastase (MacDonald *et al.*, 1982b). Very likely the activation or propeptide sequence of human renal prokallikrein is linked to the active enzyme through an Arg⁻¹-Ile⁺¹ bond, since isoleucine is the *N*-terminal amino acid of purified tissue kallikrein, followed by Val² (Lottspeich *et al.*, 1979).

Because renal prokallikrein extracted from particulate fractions was activated by thermolysin, an enzyme which cleaves at the *N*-terminal end of hydrophobic amino acids (Noda *et al.*, 1985; Matsubara & Feder, 1971), we tested thermolysin as a prototype enzyme for activation of homogeneous prokallikrein by a neutral metalloproteinase. Indeed, on a molar basis, thermolysin was three times more effective than trypsin in liberating active human renal kallikrein. A catalytic amount of thermolysin (1:50 molar ratio with prokallikrein) activated 50% in 30 min. As added active human urinary kallikrein was inactive, it is unlikely that activation of prokallikrein would involve a cascade where the release of a trace of active urinary kallikrein autoactivates prokallikrein.

Thermolysin (Matsubara & Feder, 1971) was purified from bacteria, but an enzyme that cleaves similar substrates *in vitro* and is inhibited by the same inhibitors has been purified from mammalian tissues (Kerr & Kenny, 1974; Schwartz *et al.*, 1981; Matsas *et al.*, 1983; Relton *et al.*, 1983), including the human kidney (Gafford *et al.*, 1983). This enzyme, endopeptidase-24.11, however, appears to have a more restricted active site, because the B-chain of insulin is among its higher-molecular-mass substrates; thus it may not cleave proteins. However, various mammalian cells contain other metalloproteinases, although they are not as well characterized as the endopeptidase-24.11. These enzymes resemble thermolysin and appear to be involved in important functions. These include neuromuscular transmission (Baxter *et al.*, 1983), myoblast fusion (Couch & Strittmatter, 1984) and mast-cell exocytosis (Mundy & Strittmatter, 1985). Their role in the activation of proenzymes needs to be determined.

It is of interest that plasma kallikrein activates urinary prokallikrein. Glandular kallikrein (and possibly prokallikrein as well) released from salivary gland (Rabito *et al.*, 1982, 1983) or from the basolateral membrane of the distal tubules of the kidney (Yamada *et al.*, 1981; Figueroa *et al.*, 1983) appears in circulating blood. Free kinins in plasma may be released from the low-molecular-mass kininogen by glandular-tissue kallikrein (Scicli *et al.*, 1983). Thus, conditions which lead to activation of

plasma prokallikrein (Kaplan *et al.*, 1983), such as blood clotting or thrombus formation in tissues, may result in the activation of a tissue prokallikrein as a consequence.

We are very grateful for the skilled assistance of Mr. Stephen Brown, Mr. Richard Davis and Miss Mary Fields. We thank Dr. Raymond MacDonald for his useful suggestions and Mr. Kelly Montgomery for help in preparation of the manuscript. These studies were supported in part by a grant (no. HL 20594) from the National Institutes of Health.

REFERENCES

- Abe, K., Sato, M., Imai, Y., Haruyama, T., Sato, K., Hiwatari, M., Kasai, Y. & Yoshinaga, K. (1981) *Kidney Int.* **19**, 869–880
- Baxter, D. A., Johnston, D. & Strittmatter, W. J. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4174–4178
- Corthorn, J., Imanari, T., Yoshida, H., Kaizu, T., Pierce, J. V. & Pisano, J. J. (1979) *Adv. Exp. Med. Biol.* **120B**, 575–579
- Couch, C. B. & Strittmatter, W. J. (1984) *J. Biol. Chem.* **259**, 5396–5399
- Elliot, A. H. & Nuzum, F. R. (1934) *Endocrinology* **18**, 462–474
- Fiedler, F. (1979) *Handb. Exp. Pharmacol.* **25** (Suppl.), 103–161
- Figueroa, C. C., Caorsi, I., Subiabre, J. & Vio, C. P. (1983) *J. Histochem. Cytochem.* **32**, 117–121
- Gafford, J. T., Skidgel, R. A., Erdős, E. G. & Hersh, L. B. (1983) *Biochemistry* **22**, 3265–3271
- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138
- Habener, J. F., Rosenblatt, M. & Potts, J. T., Jr. (1984) *Physiol. Rev.* **64**, 985–1053
- Hall, E. R., Kato, J., Erdős, E. G., Robinson, C. J. G. & Oshima, G. (1976) *Life Sci.* **18**, 1229–1303
- Hartley, B. S. (1970) *Biochem. J.* **119**, 805–822
- Ikekita, M., Tsuji, T., Yamamoto, K., Osawa, T., Kizuki, K. & Moriya, H. (1983) *Chem. Pharm. Bull.* **31**, 1052–1058
- Imai, T., Miyazaki, H., Hirose, S., Hori, H., Hayashi, T., Kageyama, R., Ohkubo, H., Nakanishi, S. & Murakami, K. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 7405–7409
- Kaplan, A. P., Dunn, J. T. & Silverberg, M. (1983) *Adv. Exp. Med. Biol.* **156A**, 45–61
- Kerr, M. A. & Kenny, A. J. (1974) *Biochem. J.* **137**, 477–488
- Kraut, H., Frey, E. K. & Werle, E. (1930) *Hoppe-Seyler's Z. Physiol. Chem.* **192**, 1–21
- Kraut, J. (1971) *Enzymes* 3rd Ed. **3**, 165–183
- Laemmli, U. K. (1970) *Nature (London)* **22**, 680–685
- Lottspeich, F., Geiger, R., Henschen, A. & Kutzbach, C. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1947–1950
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lundgren, S., Ronne, H., Rask, K. & Peterson, P. A. (1984) *J. Biol. Chem.* **259**, 7780–7784
- MacDonald, R. J., Sary, S. J. & Swift, G. H. (1982a) *J. Biol. Chem.* **257**, 9724–9732
- MacDonald, R. J., Swift, G. H., Quinto, C., Swain, W., Pictet, R. L., Nikovits, W. & Rutter, W. J. (1982b) *Biochemistry* **21**, 1453–1463
- Margolius, H. S. (1980) *Prog. Biochem. Pharmacol.* **17**, 116–122
- Margolius, H. S. (1984) *Annu. Rev. Physiol.* **46**, 309–326
- Matsas, R., Fulcher, I. A., Kenny, A. J. & Turner, A. J. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3111–3115
- Matsubara, H. & Feder, J. (1971) *Enzymes* 3rd Ed. **3**, 721–795
- Morichi, S., Sako, E., Hasegawa, E., Suyama, T. & Moriya, H. (1984) *Chem. Pharm. Bull.* **32**, 1152–1162
- Morita, T., Kato, H., Iwanaga, S., Takada, K., Kimura, T. & Sakakibara, S. (1977) *J. Biochem. (Tokyo)* **82**, 1495–1498
- Movat, H. Z. (1979) *Handb. Exp. Pharmacol.* **25** (Suppl.), 1–89
- Mundy, D. I. & Strittmatter, W. J. (1985) *Cell* **40**, 645–656
- Nasjletti, A. & Malik, K. J. (1981) *Annu. Rev. Physiol.* **43**, 597–609
- Nishimura, K. & Erdős, E. G. (1980) in *Enzymatic Release of Vasoactive Peptides* (Gross, F. & Vogel, G., eds.), pp. 225–234, Raven Press, New York
- Nishimura, K., Alhenc-Gelas, F., White, A. & Erdős, E. G. (1980a) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4975–4978
- Nishimura, K., Ward, P. & Erdős, E. G. (1980b) *Hypertension* **2**, 538–545
- Nishimura, K., Shimizu, H. & Kokubu, T. (1983) *Hypertension* **5**, 205–210
- Noda, Y., Takada, Y. & Erdős, E. G. (1985) *Kidney Int.* **27**, 630–635
- Nolly, H. & Lama, M. C. (1981) *Hypertension* **3**, II-35–II-38
- Ouchterlony, Ö. & Nilsson, L. A. (1981) in *Handbook of Experimental Immunology* (Weir, D. M., ed.), 3rd edn, pp. 19.13–19.21, Blackwell Scientific Publications, Oxford
- Oza, N. B., Lieberthal, W., Bernard, D. B. & Levinsky, N. G. (1981) *J. Immunol.* **126**, 2361–2364
- Rabito, S. F., Scicli, A. G., Kher, V. & Carretero, O. A. (1982) *Am. J. Physiol.* **242**, H602–H610
- Rabito, S. F., Ørstavik, T. B., Scicli, A. G., Shork, A. & Carretero, O. A. (1983) *Circ. Res.* **52**, 635–641
- Relton, J. M., Gee, N. S., Matsas, R., Turner, A. J. & Kenny, A. J. (1983) *Biochem. J.* **21**, 519–523
- Schalekamp, M. A. D. H. & Derkx, F. H. M. (1981) *Clin. Sci.* **61**, 15–21
- Schwartz, J.-C., Malfroy, B. & La Baume, S. D. (1981) *Life Sci.* **29**, 1715–1740
- Scicli, A. G., Gandolfi, R. & Carretero, O. A. (1978) *Am. J. Physiol.* **234**, F36–F40
- Scicli, A. G., Ørstavik, T. B., Rabito, S. F., Murray, R. D. & Carretero, O. A. (1983) *Hypertension* **5**, I-101–I-106
- Sealey, J. E. & Atlas, S. A. (1984) *Hypertension* **2** (Suppl. I), 115–123
- Seki, T., Nakajima, T. & Erdős, E. G. (1972) *Biochem. Pharmacol.* **21**, 1227–1235
- Silver, M. R., Ole-Moi Yoi, O., Austen, K. F. & Spragg, J. (1980) *J. Immunol.* **124**, 1551–1555
- Skidgel, R. A., Davis, R. M. & Erdős, E. G. (1984) *Anal. Biochem.* **140**, 520–531
- Spragg, J. (1983) *Adv. Exp. Med. Biol.* **156A**, 393–398
- Swift, G. H., Dagorn, J.-C., Ashley, P. L., Cummings, S. W. & MacDonald, R. J. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7263–7267
- Takaoka, M., Akiyama, H., Ito, K., Okamura, H. & Morimoto, S. (1982) *Biochem. Biophys. Res. Commun.* **109**, 841–847
- Vio, C. P., Roblero, J. S. & Croxatto, H. R. (1981) *Clin. Sci.* **61**, 241–243
- Vio, C. P., Churchill, L., Rabito, S. F., Terragno, A., Carretero, O. A. & Terragno, N. A. (1983) *Adv. Exp. Med. Biol.* **156B**, 897–905
- Vogel, R. (1979) *Handb. Exp. Pharmacol.* **25** (Suppl.), 163–225
- Ward, P. E., Gedney, C. D., Dowben, R. M. & Erdős, E. G. (1975) *Biochem. J.* **151**, 755–758
- Ward, P. E., Erdős, E. G., Gedney, C. D., Dowben, R. M. & Reynolds, R. C. (1976) *Biochem. J.* **157**, 643–650
- Werle, E. (1960) in *Polypeptides which Affect Smooth Muscles and Blood Vessels* (Schachter, M., ed.), pp. 199–209, Pergamon Press, Oxford
- Werle, E. & Urhahn, K. (1940) *Biochem. Z.* **304**, 387–396
- Werle, E. & Vogel, R. (1960) *Arch. Int. Pharmacodyn.* **126**, 171–186
- Werle, E. & Vogel, R. (1961) *Arch. Int. Pharmacodyn.* **131**, 257–261
- Yamada, K. & Erdős, E. G. (1982) *Kidney Int.* **22**, 331–337
- Yamada, K., Schulz, W. W., Page, D. S. & Erdős, E. G. (1981) *Hypertension* **3**, II-59–II-64