identity of the phenothiazine and phosphodiesterase binding sites on calmodulin remain to be identified.

Registry No. 1·HCl, 3763-80-2; 2, 87508-98-3; 3, 87508-99-4; 4, 87509-00-0; 5, 117-89-5; thiophosgene, 463-71-8; cyclic nucleotide phosphodiesterase, 9040-59-9; Ca, 7440-70-2; cAMP, 60-92-4.

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Articles

Rat Kidney Renin and Cathepsin D: Purification and Comparison of Properties†

Amintas F. S. Figueiredo,‡ Yukio Takii,§ Hiroshi Tsuji, Keitaro Kato, and Tadashi Inagami*

ABSTRACT: Renin and cathepsin D were purified by seven-step procedures involving five steps common to both enzymes. These common five steps were extraction of freeze-dried kidney powder in 30% methoxyethanol–water, diethylaminoethyl-cellulose (DEAE-cellulose) batch adsorption and elution, pepstatin–aminohexyl-Sepharose chromatography, Sephadex G-100 chromatography, and DEAE-cellulose chromatography. The renin component was purified further by passage through an anti-rat spleen cathepsin D immunoglobulin G–Sepharose (IgG–Sepharose) column followed by carboxymethyl-Sepharose (CM-Sepharose) chromatography which separated two renin components. Cathepsin D activity obtained by the fifth step was purified by passage through an anti-rat kidney renin IgG–Sepharose column followed by DEAE-Sephacel chromatography which separated three cathepsin D components. The homogeneity of renin and cathepsin D preparations was demonstrated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. The two components of renins showed molecular weights of 42000 and 36000 by gel filtration and 38000 and 36000 by SDS gel electrophoresis, respectively. They showed isoelectric points of 5.35 and 5.65 by electrofocusing in 5% polyacrylamide gels. Their optimum pHs of enzyme activity were 6.5 as determined by using nephrectomized rat plasma as a substrate. Their specific angiotensin I (Ang I) generation activities were 158 and 146 μg of Ang I (μg of protein)$^{-1}$ h^{-1}, respectively, which correspond to 1100 and 1020 Goldblatt units (mg of protein)$^{-1}$ h^{-1}. The three cathepsins showed molecular weights of 41 000, 43 000, and 41 000 by gel filtration and 46 000, 45 000, and 46 000 by SDS gel electrophoresis. They showed isoelectric points of 6.20, 6.05, and 6.00 by electrofocusing in 5% polyacrylamide gels. Each of the cathepsins showed two pH optima of 3.0 and 4.5 as examined by using bovine hemoglobin labeled with [^{14}C]-glycine methyl ester as substrate. The cathepsins showed an optimal angiotensin I generating activity of 0.98, 1.27, and 1.22 μg of Ang I (μg of protein)$^{-1}$ h^{-1}, respectively, at pH 4.5. However, at pH 6.5, the cathepsin D showed a much diminished renin-like angiotensin I generation activity.

Renin (EC 3.4.99.19) is a peptidase whose function is dedicated to the formation of decapeptide angiotensin I from its prohormone angiotensinogen by the cleavage of the singular Leu–Leu (Skeggs et al., 1957) or Leu–Val (Tewksbury et al., 1981) peptide bond located in the amino-terminal region of this substrate molecule. The discovery that the active site of renin shares many structural features commonly found in aspartic (acid) proteinases such as pepsin (Inagami et al., 1974; McKown & Gregerman, 1975; Misono & Inagami, 1980) and yet has little or practically no general protease activity has aroused interest concerning the mechanism underlying its exceedingly stringent substrate specificity.

The lysosomal aspartic proteinase cathepsin D (EC 3.4.23.5) of the brain and spleen, in a partially purified form, has been reported to possess a renin-like activity in catalyzing the formation of angiotensin I from angiotensinogen (Day & Reid,

† From the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232 (A.F.S.F., Y.T., and T.I.), and the Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan (H.T. and K.K.). *Received May 9, 1983.* Supported by Research Grants HL-14192, HL-22288, and HL-24112 and Fogarty International Fellowship TW-03014 to A.F.S.F. and by a Grant-in-Ride for Scientific Research from the Ministry of Education of Japan.

‡ Present address: Department of Biochemistry, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Brazil.

§ Present address: Department of Agricultural Chemistry, Kyoto Prefectural University, Kyoto, Japan.

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1976; Hackenthal et al., 1978). Although this activity is considered as a nonspecific action presumably without physiological significance, similarities in molecular properties of renin and cathepsin D present difficulties in the purification of renin completely free from the contamination of cathepsin D. It also presents serious problems in distinguishing true renin activity from the nonspecific action of cathepsin D in tissue extracts. In order to assess differences and similarities of renin and cathepsin D, we have undertaken purification of cathepsin-free renin and renin-free cathepsin D from the rat kidney and compared the angiotensin I forming activity of the two enzymes as well as overall molecular properties.

Experimental Procedures

Renin Activity Determination. Renin activity was determined by using unfractionated plasma of male rats as substrate. These rats had been nephrectomized bilaterally 48 h before bleeding. Blood was collected under pentobarbitol anesthesia by a cannula in the abdominal aorta. The enzymatic reaction mixture for renin contained 5 μL of a 2.5% (w/v) ethanolic solution of (PMSF)[1] (Sigma Chemical Co.), 10 μL of 200 mM Na_2EDTA, 100 μL of the nephrectomized rat plasma adjusted to pH 6.5, 110 μL of 200 mM Mes (Sigma Chemical Co.), pH 6.5, and 25 μL of an appropriate renin solution. The mixture was incubated for 15 min at 37 °C. The generated angiotensin I was then measured by radioimmunoassay according to Haber et al. (1969).

Cathepsin D Activity. Cathepsin D activity was determined by using bovine hemoglobin labeled with [^{14}C]glycine methyl ester as substrate according to Williams & Lin (1971). The enzymatic reaction was allowed to last for 10 min at 37 °C in a mixture containing 250 μL of 200 mM sodium acetate, pH 4.50, 25 μL of the substrate solution, and 25 μL of an appropriate solution of cathepsin D.

Protein Determination. Protein concentrations were determined spectrophotometrically by the method of Lowry et al. (1951) with bovine serum albumin (Sigma) as the standard. Relative protein concentrations in the chromatographic eluates were estimated by reading the optical absorbance at 280 nm.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed in 10% acrylamide (Bio-Rad) gels containing 0.1% SDS (Bio-Rad) according to the method of Laemmli (1970). Samples were dissolved in a mixture containing 15.5% (v/v) mercaptoethanol (Aldrich) and 2.7% SDS by heating for 2 min in a boiling water bath. Proteins in the gel were stained with Coomassie blue R250 (Bio-Rad). Proteins used as molecular weight standards (Bio-Rad) were phosphorylase *b* (M_r 92 500), bovine serum albumin (M_r 66 200), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), SBTI (M_r 21 500), and hen egg white lysozyme (M_r 14 400).

Isoelectric Focusing. Isoelectric points were determined by electrofocusing renin and cathepsin at 200 V for 18 h at room temperature in 5% polyacrylamide gels with 2.5% cross-linkage containing 5% ampholine (LKB), pH 4.5–7.0. Ten-centimeter gel strips were cut to 2.5-mm slices and extracted overnight either with distilled water or with 10 mM Tris-HCl, pH 7.4, containing 0.1% (w/v) bovine serum albumin. The pH of the extract was determined by glass electrodes, and the renin and cathepsin D activities were determined as described above.

Analytical Gel Filtration. The apparent molecular weights of renins and cathepsins D were determined by gel filtration on a column of Sephadex G-100 superfine (Pharmacia) (1.0 × 90 cm) which was eluted with 20 mM pyrophosphate buffer, pH 7.1, containing 100 mM NaCl, 0.1% lysozyme, and 0.2% NaN_3 (Sigma). BSA, ovalbumin, and SBTI, labeled with [^{14}C]formaldehyde by reductive alkylation with sodium cyanoborohydride in 6 M guanidine (Jentoft & Dearborn, 1979), were added to the sample solutions as internal molecular weight standards. Fractions of 800 μL were collected. The radioactivity was measured by scintillation counting.

Optimum pH of Renins and Cathepsin D. The pH dependence of renins and cathepsin D was determined by using the following buffers (0.2 M each): glycine hydrochloride for pH 2.0, 2.5, 3.0, and 3.5; sodium acetate for pH 4.0, 4.5, and 5.0; Mes for pH 5.5, 6.0, and 6.5; Tes (Sigma) for pH 7.0; and Tris-HCl for pH 7.5 and 8.0. All the buffers contained 0.1% (w/v) NaN_3. The incubations were carried out as described above.

Determination of Goldblatt Unit Values. Goldblatt unit values for renin preparations were determined at pH 6.5 by using nephrectomized rat plasma as substrate added to a kinetically saturating level in reference to the previously obtained specific activity (Matoba et al., 1978). The incubation mixture contained 500 μL of nephrectomized rat plasma adjusted to pH 6.5, 6 μL of 0.2 M Na_2EDTA, 5 μL of 5% PMSF (in absolute ethanol), and 50 μL of renin I (R-I) and renin II (R-II) diluted 50 000-fold. The mixture was incubated for 15 min at 37 °C. A standard value of 144 μg of angiotensin I/h was used as 1 Goldblatt unit.

Purification of Renin. All steps of this purification were carried out at 4 °C. Whole frozen rat kidneys were thawed at 4 °C, minced in an electric meat grinder, rappidly frozen with liquid N_2, and lyophilized. Dry cakes were pulverized in a Waring blender, and the resulting poweder was defatted by stirring in diethyl ether, filtered, and air-dried in a hood at room temperature. The powder (500 g) was extracted by stirring for 2 h in 5 L of 10 mM sodium phosphate buffer, pH 6.5, containing 0.5 mM sodium tetrathionate, 0.5 mM Na_2-EDTA, 0.08 mM PMSF, 0.1 mM DFP (Sigma), and 30% (v/v) 2-methoxyethanol. The insoluble residue was separated by centrifugation at 9000 rpm for 30 min followed by filtration through layers of gauze. The residue was reextracted by stirring for 1 h in 3 L of the same buffer. The supernatants from the two extraction steps (8 L) were combined (crude extract) and mixed with 1500 g (wet cake) of DEAE-cellulose (Whatman DE-52), the pH of the mixture was adjusted to 6.5, and the mixture was stirred gently for 12 h. The DEAE-cellulose was collected by filtration and washed in a Büchner funnel with cold distilled water. Renin was eluted by stirring the DEAE-cellulose for 2 h with 5 L of 10 mM phosphate, pH 6.5, containing 0.5 mM sodium tetrathionate, 0.5 mM Na_2EDTA, 0.08 mM PMSF, 0.1 mM DFP, 3% (v/v) 2-methoxyethanol, and 0.1 M NaCl. The DEAE-cellulose was separated by filtration. The elution was repeated by stirring in 3 L of the same buffer for 1 h. Eluates were combined (8 L), and NaCl (420 g) was added to 1 M. The pH of the mixture was adjusted to 6.5, and then the mixture was centrifuged at 12 000 rpm for 30 min to remove precipitates.

The clear eluate (8100 mL) from DEAE-cellulose in a batch was applied to a column (5 × 20 cm, 390 mL) of pepstatin–aminohexyl-Sepharose (Murakami & Inagami, 1975) previ-

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; DFP, diisopropyl phosphorofluoridate; SDS, sodium dodecyl sulfate; SBTI, soybean trypsin inhibitor; DEAE, diethylaminoethyl; CM, carboxymethyl; IgG, immunoglobulin G; Ang I, angiotensin I; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; Cath, cathepsin.

Table I: Purification of Rat Kidney Renin

purification steps	total protein (mg)	sp act. [μg of Ang I (μg of protein)$^{-1}$ h^{-1}]	total activity (μg of Ang I/h)	x-fold purification	yield (%)
(1) crude extract	83 600	0.018	1 510 000	1	100
(2) DEAE-cellulose batch	31 700	0.030	952 000	1.7	63.2
(3) pepstatin–aminohexyl-Sepharose	31.8	15.8	502 000	879	33.4
(4) Sephadex G-100	4.66	86.1	401 000	4780	26.6
(5) DEAE-cellulose	3.20	110	353 000	6130	23.5
(6) anti-rat spleen cathepsin D IgG–Sepharose	3.00	112	336 000	6230	22.3
(7) CM-Sepharose					
R-I	1.34	140	188 000	7790	12.5
R-II	1.03	116	119 000	6430	7.9
total of R-I and R-II	2.37		308 000		20.2

ously equilibrated in 10 mM phosphate, pH 6.5, containing 5 mM sodium tetrathionate, 5 mM Na_2EDTA, 0.08 mM PMSF, 3% (v/v) 2-methoxyethanol, and 1.0 M NaCl. The column was washed exhaustively with the equilibration buffer and then eluted with 0.1 M Tris-HCl, pH 7.5, containing 5 mM sodium tetrathionate, 5 mM Na_2EDTA, 0.08 mM PMSF, and 3% (v/v) 2-methoxyethanol (fraction volume 15.5 mL).

Renin-containing fractions were pooled, dialyzed against 10 mM pyrophosphate, pH 6.5, containing 0.5 mM Na_2EDTA, 0.1 M NaCl, and 0.05% (w/v) NaN_3, and then concentrated to 25 mL by pressure filtration on a YM-10 membrane (Amicon). In was centrifuged to remove precipitates at 10 000 rpm for 15 min and applied to a Sephadex G-100 superfine column (5 × 100 cm) equilibrated in 10 mM pyrophosphate, pH 6.5, containing 0.5 mM Na_2EDTA, 0.1 M NaCl, and 0.5% (w/v) NaN_3 (fraction volume 16 mL).

Renin-containing fractions were pooled and dialyzed against 20 mM phosphate, pH 6.5, containing 0.5 mM Na_2EDTA. After dialysis, the solution was concentrated by pressure filtration to 18.5 mL, centrifuged at 10 000 rpm for 30 min, and applied to a DEAE-cellulose column (1.5 × 30 cm) equilibrated with the 20 mM phosphate buffer, pH 6.5, containing 0.5 mM Na_2EDTA, pH 6.5. The column was washed thoroughly with the equilibration buffer and eluted with the same buffer containing 0.1 M NaCl. Fractions of 4 mL were collected. The pass-through fractions which contained cathepsin D and later fractions containing renin were pooled separately. The renin pool was concentrated to 20 mL as described above.

The concentrated solution was mixed with 2 mL of anti-rat spleen cathepsin D IgG–Sepharose equilibrated with 50 mM phosphate, pH 7.0, containing 0.15 M NaCl. The mixture was stirred for 18 h and poured into a column (3 mL). The column was washed with the equilibration buffer followed by 0.1 M glycine hydrochloride, pH 3.0 (fraction volume 4 mL). The pass-through fractions and washings, which contained renin, were pooled and dialyzed against 30 mM sodium acetate, pH 4.6, containing 0.5 mM Na_2EDTA.

The dialyzed solution (35 mL) was centrifuged at 10 000 rpm for 30 min and applied to a CM-Sepharose CL-4B (Sigma) column (1.5 × 30 cm) equilibrated with 30 mM sodium acetate, pH 4.6, containing 0.5 mM Na_2EDTA. The column was washed with the equilibration buffer and then eluted with 200 mL of a linear gradient of 0.15 M NaCl in the equilibration buffer (fraction volume 3.5 mL). The fractions which contained renin were pooled and stored at 4 °C.

Purification of Cathepsin D. The cathepsin D pool from three DEAE-cellulose chromatographies was concentrated to 11 mL by pressure filtration on a YM-10 membrane. It was applied to an anti-rat kidney renin IgG–Sepharose column (2 × 25 cm) equilibrated with 50 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl. The column was eluted with the equilibration buffer. It was regenerated by washing with 0.1 M glycine hydrochloride, pH 3.0 (fraction volume 2.5 mL). Cathepsin D containing fractions were pooled and dialyzed against 5 mM phosphate, pH 7.0.

The dialyzed solution was centrifuged at 10 000 rpm for 30 min and applied to a DEAE-Sephacel (Sigma) column (10 mL) equilibrated with 5 mM phosphate, pH 7.0. The column was eluted by a stepwise increase of NaCl concentration from 0, 20, and 50 mM in 5 mM phosphate buffer, pH 7.0 (fraction volume 2.5 mL). Cathepsin D containing fractions were pooled and stored at 4 °C.

Results

Purification of Renin. Results of the purification of renin are summarized in Table I. By the seven-step procedure, a 10 000-fold purification was attained with a 20% yield. Starting with 500 g of the dry rat kidney powder, derived from approximately 7500 rat kidneys (3.3 kg), we obtained 2.3 mg of renin. Two renin peaks were isolated which were stable for at least 4 months at pH 4.6 and 4 °C.

As shown in Table I, the pepstatin–aminohexyl-Sepharose chromatography (step 3) was one of the most important steps in this purification procedure which accomplished a 500-fold purification in a single step. Both renin and cathepsin D were eluted by 0.1 M Tris-HCl, pH 7.5. However, the latter was eluted in a broader trailing peak, and it was possible to separate a large amount of cathepsin D from renin by this step, although renin was not completely free from the contamination of cathepsin D.

The subsequent DEAE-cellulose chromatography (step 5) purified renin only 1.3 times. However, this step was essential for the removal of most of cathepsin D which had been copurified up to the previous step. Most of cathepsin D passed through the column whereas renin was adsorbed to DEAE-cellulose at this pH.

The small amount of cathepsin D which had been copurified with renin was completely removed by the subsequent chromatography on a small column of Sepharose coupled with anti-rat spleen cathepsin D IgG (step 6). This chromatographic step did not improve the extent of purification as no other components were removed in an appreciable quantity (Table I).

CM-Sepharose chromatography (step 7) contributed an additional 1.5-fold purification. As shown in Figure 1, two renin components were separated.

Purification of Cathepsin D. Results of the purification of cathepsin D are summarized in Table II. By the seven-step procedure, a 3250-fold purification was attained. A small

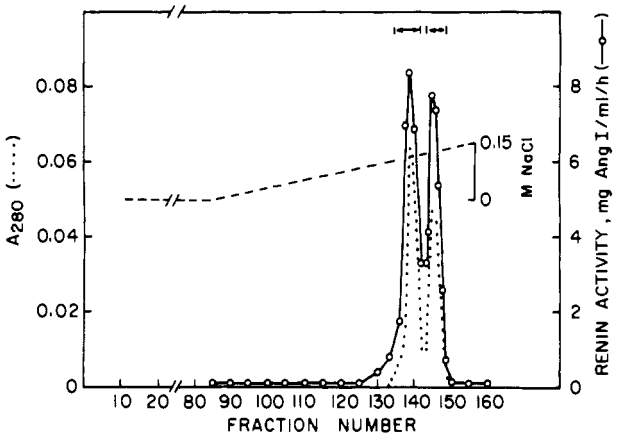

FIGURE 1: CM-Sepharose column chromatography of the renin pool from anti-rat spleen cathepsin D IgG–Sepharose chromatography. The inclinated line indicates the linear gradient of 0.15 M NaCl. Renin (O) activity is described under Experimental Procedures.

Table II: Purification of Rat Kidney Cathepsin D

purification steps	sp act. [cpm (μg of protein)$^{-1}$ h^{-1}]	x-fold purification
(1) crude extract	3.02	1
(2) DEAE-cellulose batch	6.95	2.3
(3) pepstatin-aminohexyl-Sepharose 189.7	190	62.8
(4) Sephadex G-100	666	221
(5) DEAE-cellulose	2850	944
(6) anti-rat kidney renin IgG–Sepharose	3250	1080
(7) DEAE-Sephacel		
Cath D-I	5180	1710
Cath D-II	8670	2870
Cath D-III	9810	3250

Table III: Comparison between Some Physicochemical Properties of Rat Kidney Renin and Cathepsin D

enzyme	M_r gel filtration	M_r SDS electrophoresis	pI
renin I	39 000	38 000	5.35
renin II	35 500	36 000	5.65
Cath D-I	40 740	46 000	6.20
Cath D-II	42 660	45 000	6.05
Cath D-III	40 740	46 000	6.00

amount of renin passed through the DEAE-cellulose chromatography with cathepsin D, but it was completely removed by the subsequent chromatography on a column of Sepharose coupled with anti-rat kidney renin IgG (step 6, Table II). DEAE-Sephacel chromatography (step 7) contributed an additional 3-fold purification. The purification of cathepsin D was carried out without regard to yield. Rather, fractions which copurified with renin were collected up to the step of pepstatin–Sepharose affinity chromatography in order to demonstrate copurification and contamination of these two enzymes. Therefore, Table II does not contain columns indicating yields and the total amount of cathepsin activity at each step. As shown in Figure 2, three cathepsin D components were separated. These preparations were stable at least for 4 months stored at pH 7 and 4 °C.

Molecular Properties. Results of the determination of molecular properties of rat renal renin and rat renal cathepsin D are summarized in Table III. Molecular weight values of renin determined by the two different methods, gel filtration

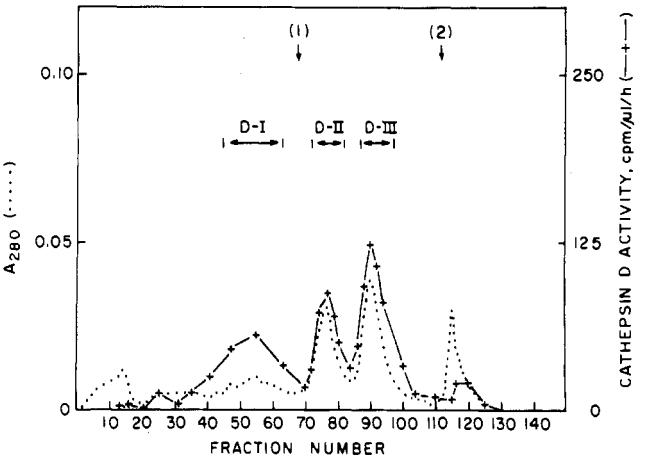

FIGURE 2: DEAE-Sephacel column chromatgraphy of the cathepsin D pool from anti-rat kidney renin IgG–Sepharose chromatography. The arrows and the numbers indicate the beginning of elutions with (1) 5 mM phosphate, pH 7.0, (2) 5 mM phosphate, pH 7.0, containing 0.02 M NaCl, and finally with 0.05 M phosphate, pH 7.0, containing 0.05 M NaCl. Cathepsin D (+) activity is described under Experimental Procedures.

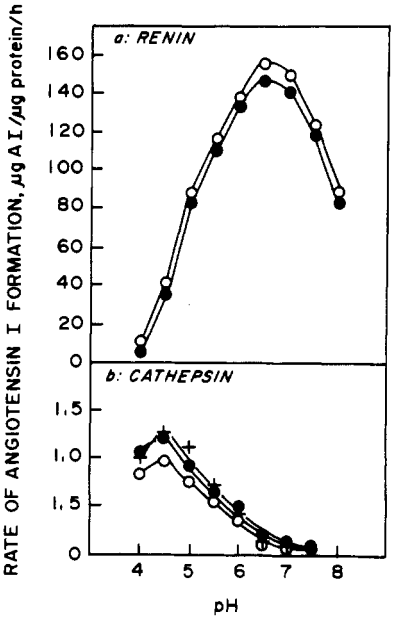

FIGURE 3: Comparison of the pH dependence of angiotensin I forming activity of renins and cathepsins purified from rat kidney. Panel a shows pH profiles of renin I (O) and renin II (●). Panel b shows pH profiles of cathepsin D-I (O), D-II (●), and D-III (+).

and SDS gel electrophoresis in the presence of mercaptoethanol, agree reasonably well. On the other hand, the molecular weights of cathepsin D determined by these methods show approximately 10% differences, the gel filtration method giving lower values for all three isoenzymes.

Isoelectric points of cathepsin D were found to be higher than those of renin by 0.3–0.8 pH unit, presumably providing the basis for partial separation of renin and cathepsin D by ion-exchange chromatography on DEAE-cellulose.

Enzymatic Properties. The pH optima of enzyme activity of renins I and II were both 6.5 (Figure 3a). Specific activities at this pH were 158 μg of angiotensin I formed (μg of enzyme)$^{-1}$ h^{-1} for renin I and 146 μg of angiotensin I formed (μg of enzyme)$^{-1}$ h^{-1} for renin II at this pH. The substrate concentrations used were such that the rate of angiotensin I generation was at the maximal level.

All three isoenzymes of cathepsin D were found to have angiotensin I forming activity. However, as shown in Figure

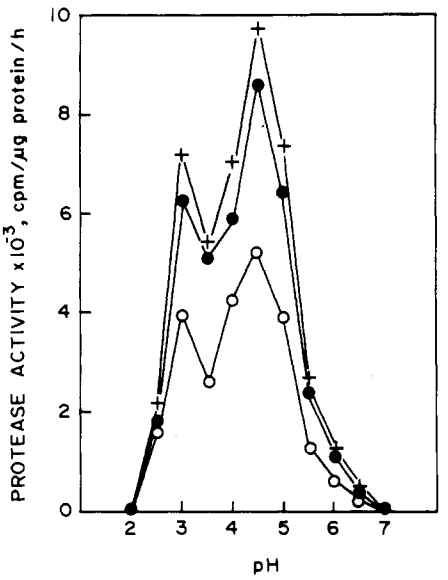

FIGURE 4: Determination of the optimum pH for cathepsin D-I (○), D-II (●), and D-III (+) with bovine hemoglobin labeled with [^{14}C]glycine methyl ester as substrate. Buffers and cathepsin D activity are described under Experimental Procedures.

3b, the pH optimum of the cathepsin-catalyzed angiotensin I generation was at pH 4.5. The specific activities of this renin-like activity of cathepsin D at pH 4.5 were 0.98 μg of angiotensin I (μg of enzyme)$^{-1}$ h^{-1} for D-I, 1.27 μg of angiotensin I (μg of enzyme)$^{-1}$ h^{-1} for D-II, and 1.22 μg of angiotensin I (μg of enzyme)$^{-1}$ h^{-1} for D-III. These specific activity values are approximately $^{1}/_{100}$th of those for renin at pH 6.5. The cathepsin-mediated activity at pH 6.5 is less than $^{1}/_{100}$th of that of renin at pH 6.5. This renin-like activity by cathepsin D was not inhibited by antibody to rat renin at 1:100 dilution. (A detailed description on the preparation of antibodies and inhibition of renin will be published elsewhere.) These observations indicated that cathepsin D exhibits intrinsic renin-like activity. The concentrations of rat plasma renin substrate used were such that the rates of angiotensin I generation by cathepsins D were at the maximal level. The hydrolysis of ^{14}C-labeled hemoglobin by cathepsins D-I, D-II, and D-III showed biphasic pH optima at pH 3.0 and 4.5 (Figure 4).

Discussion

The seven-step procedure used in this work allowed us to completely purify rat kidney renin and cathepsin D free from contamination by each other. The purification procedures followed differ notably from that described by Matoba et al. (1978).

The DEAE-cellulose adsorption by a batch process was done at pH 6.5 and the renin elution also performed at pH 6.5 with 0.1 M NaCl rather than at a lower pH. The ammonium sulfate fractionation and chromatography in a Sephadex G150 column and a CM-cellulose column at pH 5.35 were deleted. The latter procedure performed at pH 5.35 caused a drastic decrease in recovery of the enzyme presumably due to isoelectric precipitation in the column.

The procedure now described also shows a marked difference in the affinity chromatography procedure on pepstatin–aminohexyl-Sepharose column. The chromatography was run at pH 6.5 with 1.0 M NaCl instead of at pH 5.5. A much better adsorption of renin was accomplished under the present condition whereas by the previous procedure a sizable portion of renin tended to leak through the pepstatin column. The presence of 1 M NaCl seemed to minimize nonspecific binding of proteins by ionic interaction. The elution was done with 0.1 M Tris-HCl, pH 7.5, without NaCl.

As the result of this modification, the affinity chromatography afforded a 500-fold purification compared with a 40-fold purification in the previous study. Above all, the immunochromatography of renin on anti-rat spleen cathepsin D IgG–Sepharose, an important step in this work, removed the small amount of cathepsin D contamination. Finally, by the last chromatographic step on CM-Sepharose at pH 4.6, two renin components, renin I and renin II, were separated.

Previous studies on rat kidney renin (Lauritzen et al., 1976; Matoba et al., 1978) showed the presence of at least three renin components while in the present study only two components were isolated. Isoelectric focusing did not separate these components to additional subcomponents. Because the procedures used in these three works are considerably different, it is possible that a different number of renin components should be expected. The present isolation work was repeated 3 times, and the two-peak pattern was obtained persistently. Rapidity of the work compared with previous studies may account for such a difference. Prolonged manipulation of crude homogenates or extracts seems to give rise to multiple component patterns. Minor components may have been eliminated during a multistep purification procedure. Whatever the reason may be for the difference in the number of components, the results of the past and present studies indicate rat renin consists of more than a single component.

During the entire purification step, the pH was maintained at 6.5, and renin remained stable at 4 °C. Once cathepsin D was completely removed, renin showed a satisfactory stability even at pH 4.6 over a period of 4 months at 4 °C.

The pH optimum of rat renin prepared in the present study was 6.5 which was in good agreement with the previously reported range between pH 6.5 and 7.5 (Lauritzen et al., 1976; Matoba et al., 1978).

Rat kidney cathepsin D was purified through steps common with purification of renin in the early stages of the purification procedure. Since cathepsin D is also adsorbed by pepstatin–Sepharose and eluted by 0.1 M Tris-HCl at pH 7.5, renin and cathepsin D can be purified together up to this point. This property of cathepsin D indicates the danger of cathepsin D being a dominant contaminant of renin preparation unless strict precautionary measures are taken to separate these enzymes in the subsequent steps. The ion-exchange chromatography on DEAE-cellulose operated at pH 6.5 gave a good separation of these enzymes. Three subcomponents of cathepsin D were separated by the final ion-exchange chromatography on a DEAE-Sephacel column. These components are closely related with respect to their molecular weights as determined by gel filtration (41 000, 42 000, and 41 000, respectively) and by SDS–polyacrylamide gel electrophoresis (46 000, 45 000, and 46 000, respectively) as shown in Table III. The two cathepsin D components isolated from rat spleen by Yamamoto et al. (1970) showed molecular weights of 44 000 by gel filtration on Sephadex G-100 as well as by SDS–polyacrylamide gel electrophoresis, in reasonable agreement molecular weight of the present cathepsin D from rat kidneys. The p*I* values of the kidney enzyme (6.20, 6.05, and 6.00) were somewhat different from those of the spleen cathepsin D-I (4.2, 4.9, 6.1, and 6.5) and spleen cathepsin D-II (4.6, 5.6, and 5.8). Huang et al. (1979) reported six components of porcine spleen cathepsin D with p*I* values ranging from 5.51 to 7.54. Compared with these, rat renal cathepsins D seem to have a narrower range of spread in the p*I* value.

The rat renal cathepsin D showed bimodal pH dependency in its enzymatic activity with a minor pH optimum at 3.0 and the major peak at 4.5, when examined with ^{14}C-labeled denatured bovine hemoglobin as substrate. Yamamoto et al. (1979) reported single pH optima of 3.8 for rat spleen cathepsin D-I and 3.5 for D-II using bovine hemoglobin as substrate. On the other hand, Cunningham & Tang (1976) found two optima at pH 3.4 and 3.8 for porcine spleen cathepsin D. Barrett (1971) found a similar bimodal pH dependency for cathepsin D from human and chicken livers. Thus, the bimodal pH optima may be an intrinsic property of the hemoglobin–cathepsin D system presumably determined by the conformational change or partial denaturation of the enzyme and substrate. The possibility that the bimodal pH optima were due to isoenzymes seems to be eliminated as each of the isolated isoenzymes shows a similar pH profile of enzyme activity.

Noteworthy was the finding that cathepsin D from rat kidney showed a considerable renin-like activity at pH 4.5 in generating angiotensin I from rat angiotensinogen (Table III) which amounted to about $^1/_{100}$th of the activity of renin at pH 6.5. This may be due to close structural similarities of cathepsin D with renin and accounts for the strong renin-like activity of the so-called pseudorenin (Skeggs et al., 1969) which was eventually identified as a cathepsin D (Dorer et al., 1978).

At pH 6.5, where renin shows its pH optimum, cathepsins D-I, D-II, and D-III produce angiotensin I at rates approximately 3 orders of magnitude less than that of renin. This level is much higher than the estimate made by Hackenthal et al. (1978) using renin whose specific activity was not completely determined. If one may extrapolate these results to other tissues where renin concentration is much less than that in the kidney, the contribution of the renin-like activity of cathepsin D even at pH 6.5 may still be significant compared with that of renin itself. Thus, the present results indicate the need for caution in estimating the renin activity of various tissues where renin concentration is very low.

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