Biochimica et Biophysica Acta, 526 (1978) 560—571

BBA 68541

RAT RENIN: PURIFICATION AND CHARACTERIZATION

TERUYOSHI MATOBA *, KAZUO MURAKAMI ** and TADASHI INAGAMI ***

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tenn. 37232 (U.S.A.)

(Received December 15th, 1977)
(Revised manuscript received April 24th, 1978)

Summary

In order to clarify the molecular basis of the unique features of rat renin (EC 3.4.99.19) and to provide materials and basic information for high blood pressure studies in rats, renin was purified from rat kidney. The final step of purification on CM-cellulose separated renin into three major isoenzyme peaks, R-I, R-II, R-III, and an additional minor peak. These preparations were judged homogeneous by multiple criteria, and the isoenzymes were found to have similar amino acid compositions. The amino acid composition is also closely analogous to hog renin, except that rat renin has a higher cysteine content. In contrast to hog renin, the rat enzymes do not contain amino sugars, yet are apparently glycoproteins as judged by their affinity for concanavalin A. The molecular weights of R-I, R-II, and R-III were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 37 000, 36 000 and 35 000, respectively. The isoelectric points were 5.05, 5.15 and 5.22, respectively. The specific activities of the purified enzymes (determined using rat plasma as substrate) were 615, 626 and 452 Goldblatt units/mg, respectively. Comparison of activities with the hog- and rat-derived substrates indicated a preference for that from the rat. The reaction of the rat enzymes with a synthetic peptide substrate had a similar catalytic rate constant to the hog enzyme, indicating close similarity in the active site region of the two enzymes.

Introduction

Renin (EC 3.4.99.19) is an acidic protease and its only known function is to produce angiotensin I from the α_2-globulin substrate. Since it catalyzes the first

* Present address: Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan.
** Recipient of Tennessee Heart Association Investigatorship Award. Present address: Institute of Applied Biochemistry, University of Tsukuba, Ibaragi-ken, Japan.
*** To whom correspondence and reprint requests should be addressed.

step of the renin-angiotensin-aldosterone cascade, the importance of this enzyme in blood pressure regulation and certain types of hypertension is well recognized. However, studies on the biochemical properties of this enzyme have been severely hindered due to the lack of a pure preparation. Recently, renin from hog renal cortex has been purified by affinity chromatography [1]. We have also purified renin from the submaxillary gland of the mouse by a conventional technique [2]. The choice of hog kidney and mouse submaxillary gland was made because of relatively high renin content in these organs. However, pigs and mice are rarely used in studies on blood pressure regulation and hypertension.

Interest in the angiotensin system of the rat has been intensified with the advent of genetically inbred hypertensive rats [3], which have been the subject of extensive studies as a model for essential hypertension in man. Studies on rat renin have been limited to determination of plasma renin levels in relation to pharmacophysiological research [4—6]. Only very limited information is available about the biochemical properties of the rat enzyme [7]. Studies by Lauritzen et al. [8] indicated that rat renin has a unique property. Whereas renin from other species can be activated by acidification [9—12], rat kidney renin cannot be thus activated. This activation could play an important role in blood pressure regulation. Studies on this unique enzyme incapable of such activation could reveal the role of activation in homeostasis. We have now isolated a pure preparation of rat renin and elucidated some of its properties.

Methods and Materials

Renin activity. Renin activity was determined by using as substrate either partially purified hog angiotensinogen (Miles Laboratories) or rat plasma from nephrectomized male rats. Conditions such as substrate concentrations and pH were optimized for different substrates and are given in the figure legends. The rate of angiotensin I formation was determined by radioimmunoassay [13], using assay kits (Squibb and Son, Co.). Goldblatt unit values of rat renin preparations were determined in reference to standard rat renin (from Dr. E. Haas, Cleveland, Ohio), which had a specific activity of 0.11 Goldblatt unit/mg and produced 144 μg angiotensin I/h per unit from the rat plasma substrate (10% rat plasma: 0.54 μM in the substrate) at pH 6.5. The synthetic renin substrate, benzyloxycarbonyl-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-β-naphthylamide [14] (Bachem. Inc., Marina Del Ray, Calif.), was also used in the assays during the purification of renin [15].

Acid protease activity. Acid protease activity was determined using hemoglobin labeled with [^{14}C]glycine methyl ester, at pH 5.6 and 37° C according to Williams and Lin [16].

Protein concentration. Protein concentration was determined spectrophotometrically by using $E^{1\%}_{1cm} = 7.9$ at 280 nm. For very concentrated solutions, the biuret method was employed using bovine serum albumin as standard [17].

Extraction of rat renin. All steps of purification were carried out at 4° C, unless otherwise stated. Whole frozen rat kidneys were partially thawed at room temperature, minced in an electric meat grinder, rapidly refrozen with liquid N_2, lyophilized, defatted by stirring in diethyl ether, filtered and air-

dried at room temperature. The powder was extracted with stirring for 1 h in 15 l 30% (v/v) methoxyethanol containing 2 mM phenylmethanesulfonyl fluoride, 0.1 mM diisopropylphosphorofluoridate, 5 mM sodium tetrethionate and 5 mM EDTA. The extract was separated from insoluble residue by centrifugation and, after addition of 30 l water, renin was adsorbed to DEAE-cellulose (Whatman DE-52) by stirring for 1 h at pH 7.5. The cellulose was collected by filtration, washed with water and renin activity eluted first with 12 l 0.1 M sodium acetate buffer (pH 4.8)/0.2 M NaCl/0.1 mM phenylmethanesulfonyl fluoride/5 μM diisopropylphosphorofluoridate/0.25 mM sodium tetrethionate/0.25 mM EDTA and again with 8 l of the same buffer. After adjusting the pH to 5.5, the renin activity was precipitated from the combined eluate by addition of solid $(NH_4)_2SO_4$ to 75% saturation. Precipitates were collected by centrifugation and dissolved in 0.01 M sodium acetate buffer (pH 5.5) containing the protease inactivators at the same concentrations as the elution buffer. This solution was dialyzed against the same buffer for 24 h, changing the buffer every 8 h. It was then centrifuged at 8000 × *g* to remove insoluble materials.

Affinity chromatography. A column (2.5 × 20 cm) containing pepstatin-aminohexyl-agarose [18], previously equilibrated with 0.01 M sodium acetate buffer (pH 5.5), was charged with the clear dialyzate, washed successively with the same buffer and the same buffer containing 0.05 M NaCl until protein was no longer detectable in the washing buffer. The renin activity was eluted from the column with 0.1 M Tris · HCl buffer (pH 7.6)/0.1 M NaCl (Fig. 1). The fractions containing renin activity were pooled, adjusted to pH 6.5, and concentrated using an Amicon filtration apparatus (PM-10 membrane). This latter procedure was used throughout the present work for concentration and equilibration with chromatography buffers.

Gel filtration. The concentrated solution from the affinity column was further purified by gel filtration on a Sephadex G-150 column (5 × 100 cm) using 0.01 M pyrophosphate buffer (pH 6.0)/0.1 M NaCl/0.5 mM EDTA/0.05% NaN_3. As shown in Fig. 2, two peaks and a shoulder with renin activity were eluted from this column. Fractions under the major renin activity peak were pooled, concentrated and subjected to gel filtration on a Sephadex G-100

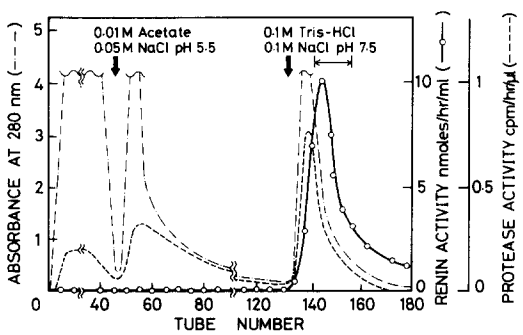


Fig. 1. Purification of rat renin using a pepstatin-aminohexyl-agarose affinity column. Renin activity was determined by the fluorometric method of Reinharz and Roth [15] using 25 μl of each fraction after incubation for 6 h at 37°C with 25 μl substrate (1 μg/ml dimethylformamide and 250 μl 0.5 M sodium pyrophosphate (pH 5.6). The acid protease activity was determined by the method of Williams and Lin [16] using 25 μl of each fraction incubated for 3.5 h at 37°C. The size of the column was 2.5 × 20 cm.

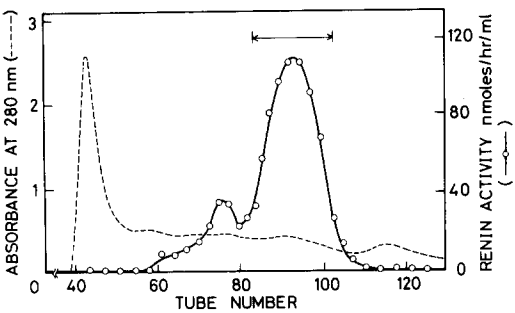
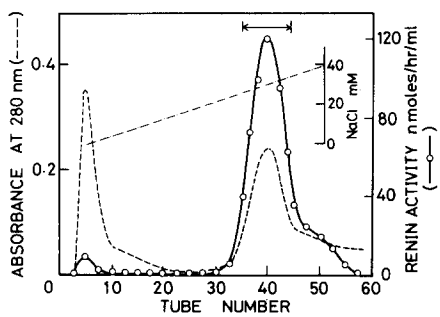


Fig. 2. Gel filtration on a Sepharose G-150 column of the rat renin fractions obtained from the affinity column. Renin activity was determined as described in Fig. 1 using 20 μl of each fraction incubated at 37°C for 1 h.

column (2.5 × 100 cm) using the same eluting buffer as in the Sephadex G-150 column.

DEAE-cellulose chromatography. The active fractions from the second gel filtration were concentrated, equilibrated with 0.02 M phosphate buffer (pH 6.5)/0.5 mM EDTA and applied to a DEAE-cellulose column (1.5 × 27 cm, Whatman DE-52) previously equilibrated with the same buffer. This column was eluted using a 600 ml linear gradient of 0—0.1 M NaCl in the same buffer (Fig. 3).

CM-cellulose chromatography. Active fractions from the DEAE-cellulose column were concentrated and equilibrated with 0.002 M sodium phosphate/0.02 M sodium acetate buffer (pH 6.5). The pH was adjusted to pH 5.35 with dilute acetic acid. This procedure was employed to minimize precipitate formation and loss of renin activity which occurred if dialysis and concentration were carried out at pH 5.35. After centrifugation at 8000 × *g* the solution was applied to a CM-cellulose column (0.9 × 52 cm, Whatman CM-52) previously equilibrated with 0.03 M sodium acetate (pH 5.35)/0.5 mM EDTA. The col-



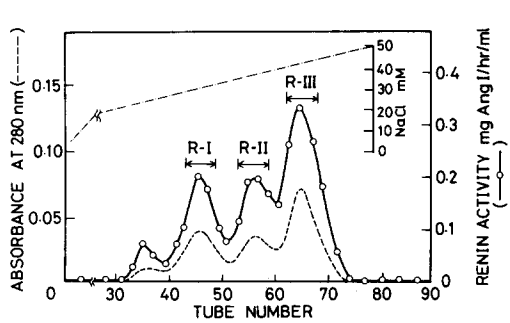


Fig. 3. DEAE-cellulose chromatography of the renin fractions from the Sephadex G-100 column. 20-μl aliquots of each fraction were incubated for 1 h at 37°C for renin activity assay as described in Fig. 1.

Fig. 4. CM-cellulose chromatography of rat renin fractions from the DEAE-cellulose column. Renin activity was assayed by determining the rate of angiotensin I formation from hog plasma angiotensinogen in 1 h at 37°C using the radioimmunoassay method of Haber et al. [13]. The renin reaction mixture, in 0.2 M Tris/maleate buffer (pH 6.0), contained 0.63 μM hog angiotensinogen and 25 μl renin solution diluted 1 : 5000 with 0.01 M Tris · HCl buffer (pH 7.5)/0.5% bovine serum albumin.

umn was eluted with a 460 ml linear gradient of 0—0.5 M NaCl in the initial buffer. Three peaks with renin activity, designated R-I, R-II and R-III, were obtained (Fig. 4).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed at pH 9.5 at 2 mA per tube in 0.5 × 6 cm columns of 7.5% polyacrylamide gel (2.5% cross-linkage) according to Davis [19] except for the omission of the stacking gel. Protein bands were visualized using Coomassie Brilliant Blue.

Molecular weight. Molecular weight was estimated both by gel filtration on a calibrated Sephadex G-150 column (1.5 × 100 cm) according to Andrews [20], and by electrophoresis on sodium dodecyl sulfate polyacrylamide gels (10% in concentration with 2.7% cross-linkage). The gels (0.5 × 10 cm) contained 0.2% SDS and were run with and without dithiothreitol (0.25%) [21,22].

Amino acid analysis. Pure renin preparations, dialyzed against 0.02 M triethylamine acetate buffer (pH 7.5) and hydrolysed in 6 M HCl for 24 h according to Moore and Stein [23], were subjected to amino acid analysis using a Durrum D500 analyzer. Cysteine was determined as cysteic acid after performic acid oxidation [24] and amino sugars were determined after 6 h hydrolysis in 4 M HCl at 100°C [25].

Isoelectric point. Isoelectric point was determined with proteins dialyzed against 1% glycine by the isoelectric focusing method [26]. The plates (10 cm) were run at 2°C for 5 h at a constant power of 15 W using a 5% polyacrylamide gel with 3% cross-linkage and containing 2.4% ampholine (pH 4—7).

Results

Purification

The purification procedure of renin from rat kidney is summarized in Table I. The present study indicated that there were at least four components in rat renin (Fig. 4); three of these (R-I, R-II and R-III) were isolated in reasonable quantities by the present purification procedures. Starting with 4.46 kg of rat kidneys, 0.46 mg of R-I, 0.42 mg R-II and 0.97 mg R-III were isolated, at a combined yield of approx. 3%. A 3000-fold purification was obtained. These preparations were used for the subsequent characterization of enzymatic properties.

The pepstatin-aminohexyl-agarose gel [18] employed for affinity chromatography removed a neutral protease activity which had accompanied the crude preparation despite repeated prior treatment with protease inactivators. The majority of the strong neutral protease activity was eluted by 0.1 M Tris · HCl buffer (pH 7.5)/0.1 M NaCl. As shown in Fig. 1, the very early fractions of renin activity overlapped with the protease peak. Therefore, in order to stabilize renin by minimizing contamination with the proteolytic enzyme, the initial renin fractions were discarded. Also, since this protease activity is suppressed to 30% original activity by 5 mM EDTA, this chelating reagent was used throughout subsequent purification steps.

Gel filtration

Gel filtration on a Sephadex G-150 column produced two renin species, one

TABLE I

PURIFICATION OF RENIN FROM RAT KIDNEY

Purification step	Total protein * (mg)	Specific activity **	Purification	Yield (%)
Crude extract ***	202 000	0.00151	1	100
Ammonium sulfate fractionation	49 500	0.00316	2.1	51.2
Affinity chromatography	646	0.122	81	25.7
Sephadex G-150	97.5	0.632	419	20.1
Sephadex G-100	61.5	0.920	609	19.6
DEAE-cellulose	8.0	3.86	2560	10.1
CM-cellulose				
R-I	0.47	4.7	3100	0.73
R-II	0.42	5.1	3400	0.70
R-III	0.97	4.1	2700	1.30
Total of R-I, R-II, R-III	1.86			2.70

* The biuret method was used for the crude extract and the ammonium sulfate fraction. The spectrophotometric method was used in all other determination using $E^{1\%}_{1cm} = 7.9$ at 280 nm.

** Determined by the rate of angiotensin formation from partially purified hog renin substrate (0.63 μM) at pH 6.0 and 37°C by the radioimmunoassay method of Haber et al. [13]. The activity is given in the unit of μg angiotensin I formed/μg protein per h.

*** Obtained from 964 g of dry, defatted powder prepared from 4.46 kg of rat kidney.

corresponding to a molecular weight of 40 000 and the other to 60 000 (Fig. 2). The latter disappeared completely when all the protease inactivators were omitted from the extraction medium. In the present study the purification was confined to the small molecular weight species.

Ion-exchange chromatography

The renin preparation after DEAE-cellulose chromatography had a specific activity almost comparable to the final preparation (Table I). No neutral or acid protease activity was detected after the DEAE-cellulose chromatography.

In all the purification steps, renin activity in eluate fractions was determined by the method of Reinharz and Roth [15] except for the final step using the CM-cellulose column. Since this method is not strictly specific for renin, results were spot checked by the radioimmunoassay method of Haber et al. [13]. Very good agreement was found between the two methods after the affinity chromatographic step.

Purity

The homogeneity of the purified rat renin preparations R-I, R-II, and R-III was demonstrated by single bands on polyacrylamide gel electrophoresis, SDS-polyacrylamide electrophoresis and electrofocusing on an ampholine-impregnated polyacrylamide gel slab. Ouchterlony double diffusion of the purified rat renin and rabbit anti-hog renin antiserum also produced a single precipitin band.

Molecular weight

Molecular weight (determined by gel filtration on a calibrated column of

TABLE II

AMINO ACID COMPOSITIONS OF RAT RENIN

Amino acids	Analysis of 24 h hydrolysate (μg/mg)			
	Rat renin			Hog renin *
	R-I	R-II	R-III	
Ala	25.1	24.4	25.3	25.6
Arg	28.3	28.8	29.2	34.2
Asp	77.4	75.8	76.8	69.4
1/2 Cys **	13.0	14.8	11.1	6.5
Glu	94.1	92.8	91.2	86.8
Gly	51.8	55.2	55.4	43.7
His	19.0	18.4	19.2	15.6
Ile	33.4	32.1	30.7	30.6
Leu	68.4	70.6	70.9	76.4
Lys	33.4	33.3	33.1	31.2
Met	17.3	18.6	18.1	11.0
Phe	54.8	53.3	57.1	58.3
Pro	37.5	38.1	38.0	38.6
Ser	61.6	58.8	56.5	57.7
Thr	53.3	52.5	53.5	60.6
Val	53.9	50.5	54.6	58.2

* Analyzed with pure hog renin prepared by the method of Inagami and Murakami [1].
** Determined as cysteic acid by the method of Hirs [24].

Sephadex G-100) was calculated at 41 000 for each of the three enzymes. Electrophoresis on SDS-polyacrylamide gel in the presence of dithiothreitol gave a molecular weight of 37 000, 36 000 and 35 000 for R-I, R-II and R-III, respectively. In the absence of dithiothreitol, the molecular weights of R-I and R-II were 39 000 and that of R-III was 37 000.

Amino acid composition

Results of duplicate amino acid analyses of the three rat isoenzymes are summarized in Table II. The amino acid compositions of the rat enzymes are also compared with that of hog renin. The very small quantities of proteins available for this study did not permit complete timed analysis and determination of tryptophan residues. However, the results indicate close similarity in the amino acid compositions of the three rat isoenzymes. Moreover, comparison with the hog enzyme, shows a close similarity in the amino acid composition of rat and hog renin. Exceptions are higher cystine/cysteine content and the almost complete lack of glucosamine in rat enzyme compared with hog renin (which contains 1.2% glucosamine).

Affinity to concanavalin A

In view of insufficient quantities of pure rat renin preparations for complete carbohydrate analysis an attempt to detect the presence of carbohydrate was made by studying the affinity of renin for concanavalin A-agarose. Renin was completely retained by a small column (0.5 × 5 cm) and was not released by 1 M NaCl, but was eluted with 0.2 M α-methyl-D-glucose (Fig. 5).

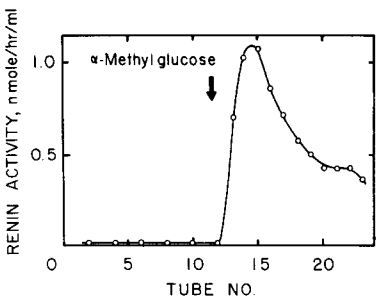


Fig. 5. Absorption of rat renin to concanavalin A-agarose gel and release by *O*-methyl-D-glucose. Renin purified through the DEAE-cellulose chromatographic step was applied to a column (0.5 X 5 cm) of concanvalin A-agarose gel (Pharmacia Fine Chemicals) equilibrated with 0.02 M phosphate buffer, pH 6.9. After exhaustive washing with the same buffer and the same buffer containing 1 M NaCl, 0.25 M *O*-methyl-D-glucose was added to the buffer to elute the retained renin activity at the point indicated by the arrow. The renin activity was determined by the fluorometric assay as described in Fig. 1.

Isoelectric point

Densitometer scans of the electrofocusing gave single peaks corresponding to isoelectric points of 5.05, 5.15 and 5.22 for R-I, R-II and R-III, respectively, in reasonable agreement with values obtained for partially purified rat renin by Lauritzen et al. [8].

Stability

Freezing and thawing caused partial inactivation of the three species of rat renin. One cycle of freezing and thawing of a 0.01% solution cuased an approx. 30% loss in activity, independent of the length and temperature of storage. Thus, storage at −20° C for 1 month did not cause more than 30% inactivation. The effect of freezing and thawing was concentration dependent. One cycle of freezing-thawing of 0.0002% solution completely inactivated the enzyme. 0.5% bovine serum albumin protected rat renin from this inactivation and the purified enzymes were stored with this protein concentration, at pH 7.0. Albumin had no effect on renin activity determined by the radioimmunoassay method of Haber et al. [13].

Enzyme activity

The pH dependency of rat renin activity was determined using three different substrates: pooled rat plasma, hog angiotensinogen and the synthetic octapetide, benzyloxycarbonyl-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-β-naphthylamide. With the rat plasma, the activity of the three isoenzymes was maximal from pH 6.0 to 6.5 at 37° C (Fig. 6, open circles). With the hog substrate, again a broad plateau of activity was observed from pH 5.5 to 6.5 for the three components (Fig. 6, filled circles). In contrast, the hydrolysis of the synthetic substrate (examined with renin purified up to the DEAE-cellulose chromatographic step) was optimal at pH 4.0—4.5 (Fig. 7). These pure rat renin preparations (5 μg) did not exhibit general acid protease activity as examined by the method described above at pH 3.5, 4.5 and 5.7, using ^{14}C-labeled hemoglobin [16].

Examination of the dependency of enzyme activity on substrate concentration indicated that 2.7 μM rat angiotensinogen (3450 ng angiotensin I equiva-

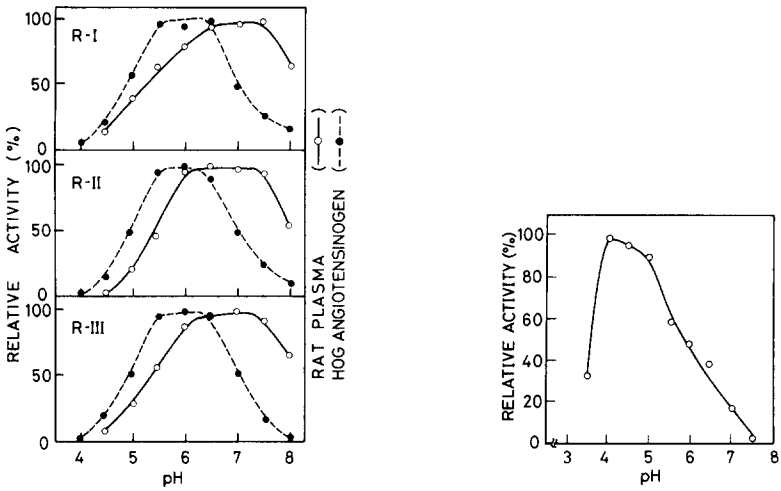


Fig. 6. The pH dependence of the renin reaction with different substrates. (a) Rat plasma renin substrate (—○—). A reaction mixture (0.5 ml) consisted of buffer (0.2 M) containing 50% plasma of nephrectomized rats and 50 μl of purified rat renins. R-I was used after a 4000-fold dilution, R-II after a 5000-fold dilution and R-III after a 7000-fold dilution. Angiotensin I formed during the 1 h reaction carried out at 37°C was determined by the radioimmunoassay method of Haber et al. [13]. (b) Hog renin substrate (—●—). The rate of formation of angiotensin I from partially purified hog plasma substrate (Miles Laboratories) added at a concentration of 9.63 μM in a 0.5 ml reaction nixture containing buffer (0.2 M) was determined by the radioimmunoassay method as described in a. Buffers used were: sodium-citrate for pH 3.5—4.0, sodium acetate for pH 4.0—5.5, 2-(N-morpholino)-ethane sulfonyl chloride for pH 5.5—6.5, morpholinopropane sulfonyl chloride for pH 6.5—7.5 and Tris · HCl for pH 7.5—8.0.

Fig. 7. The pH dependence of renin reaction with the synthetic octapeptide substrate. The substrate (2.5 μg) and renin (2.2 μg) obtained after DEAE-cellulose chromatography were allowed to react in 0.25 ml of 0.2 M buffers for 2 h at 37°C. The reaction was terminated by heating in a boiling water bath for 5 min. After addition of 0.1 ml 1 M tricine buffer, pH 7.5, containing 0.45 mM zinc acetate, β-naphthylamine was released by reaction with 0.5 unit of aminopeptidase M (Sigma Chemical Co.) at 37°C for 3.5 h and was determined fluorometrically [15]. Buffers used are identical as those described in Fig. 6.

TABLE III

SPECIFIC ACTIVITY OF RAT RENINS

Renin preparation	Specific activity of rat renins	
	Rat plasma * (Goldblatt units/mg)	Hog renin substrate **
R-I	108.5	35.0
R-II	110.1	36.3
R-III	79.8	27.9

* To 0.5 ml plasma adjusted to pH 6.5 were added 6 μl 0.2 M disodium-EDTA, 5 μl 5% (in isopropanol) diisopropylfluorophosphate and 50 μl renin (R-I diluted 4000-fold, R-II 5000-fold and R-III 7000-fold with 0.5% bovine serum albumin). After incubation at 37°C and 1 h, 10 μl was used for radioimmunoassay.

** To 0.5 ml 0.2 M sodium maleate buffer (pH 6.0) containing partially purified hog substrate (9.4 μM), 5 μl each of the EDTA and diisopropylfluorophosphate solutions, were added 10 μl diluted renin. After 1 h at 37°C, 10 μl reaction mixture was subjected to the radioimmunoassay.

*** Estimated in reference to the activity of the standardized rat renin supplied by Dr. Erwin Haas of Cleveland. The activity of the standard determined under the assay conditions employed in these experiments was 144 μg angiotensin 1/h Goldblatt unit.

lent/ml; obtained by a 2-fold dilution of rat plasma) gave a maximal rate at pH 6.5 and 37°C, whereas, at the conditions regularly used for renin assay in this study (0.5 μM, 10% (v/v) plasma) a rate approx. 30% of the maximum was obtained. With hog angiotensinogen, the rate reached a maximal level at 6.3 μM. At 0.63 μM (where the renin was routinely assayed) the activity was approx. 14% of the maximum rate. The specific activities determined at substrate concentrations which give maximal catalytic rates are shown in Table III. The results indicate that rat renin reacted with rat plasma three times more efficiently than with the hog angiotensinogen. Only slight differences were observed between the specific activities of R-I, R-II and R-III. Goldblatt unit values of these preparations (Table III) were determined at pH 6.6 by using rat plasma in reference to the standardized rat renin of Dr. Haas. The results indicate that the specific activities of R-I and R-II were similar, while that of R-III was somewhat lower. Kinetics of the hydrolysis of the synthetic octapeptide substrate was studied in 10% (v/v) dimethylformamide at 37°C and pH 4.5 (where the rate of renin catalysis on this substrate is maximal). Initial rates, determined at different substrate concentrations ranging from 11 to 54 μM, produced linear Lineweaver-Burk plots. K_m values of 30, 34 and 41 μM were obtained for R-I, R-II and R-III, respectively. V values for R-I, R-II and R-III were estimated as 0.85, 0.82 and 0.74 μmol/h per mg, respectively. Using molecular weight values estimated by SDS-polyacrylamide gel electrophoresis in dithiothreitol, the turnover numbers were computed as 0.52, 0.49 and 0.43 min^{-1}, respectively.

Discussion

The general objective of the present study was to determine the properties of rat renin and to identify those which might explain some of its unique features. A purified preparation of rat renin was used and the results may provide a frame of reference for studies on the pathophysiology of blood pressure regulation in the rat previously conducted using only impure enzymes.

Compared to hog renin, rat renin has a considerably weaker affinity for the pepstatin-aminohexyl-agarose gel. It was bound only in the mildly acidic pH range but not at a neutral pH, whereas hog renin was bound over a wide range from pH to 7.5. Rat renin could thus be eluted merely by slightly elevating the pH of the eluting buffer, rather than the drastic acidification required for the elution of hog renin [18]. In spite of the adherence to neutral pH conditions during the isolation procedure, only residual quantities of high molecular weight forms were seen upon subsequent separation by gel filtration (Fig. 2). The activity in the first minor renin peak corresponds to a molecular weight of 60 000 and a shoulder before this peak corresponds to 100 000—120 000. Analogy with hog renin [27] suggests that these high molecular weight forms could be the renin precursors with low specific activities.

The 3400-fold purification obtained between the intial extract and pure renin is low (Table I) compared with the greater than 100 000-fold purification obtained in the preparation of pure hog renin [1]. However, a direct comparison of these two numbers is misleading since the initial rat kidney extract seems to contain almost eight times as high a renin activity per unit protein

concentration as the similar hog kidney extract obtained in our previous studies. This is because the rat renin activity (Table I) was determined with heterologous hog angiotensinogen at a substrate concentration where the rate of angiotensin I generation is only 1/7 of the level of V, whereas the renin activity of the initial hog kidney powder extract was determined with its homologous substrate at a concentration where the rate is practically identical with its V [1]. Since the apparent rates of angiotensin generation determined under these conditions for the initial hog and rat kidney powder extracts are comparable [1], we can conclude that rat renin present in the initial extract was approx. 10 times as much as the hog renin in the initial extract from hog kidney powder. Thus the present 2700—3400-fold purification is equivalent to 27 000—34 000-fold purification of hog renin *.

The three major renin isoenzymes, R-I, R-II and R-III (Fig. 4) purified from rat kidney were closely analogous to each other in a number of properties. No major differences could be detected in amino acid compositions of the three isoenzymes. The absence of amino sugars is an important feature which distinguishes the rat enzyme from the hog enzyme. It should be noted that, despite the absence of detectable amino sugars, the rat enzyme seemed to be a glycoprotein as suggested by its specific affinity for concanavalin A (Fig. 5).

The optimal pH range for the enzyme activity of rat renin was dependent on substrate (Fig. 6). A similar but more pronounced dependence of pH optimum on substrate (from 5 to above 8) has been noted with mouse submaxillary gland renin [28]. Such wide variance of pH optimum seems to be a characteristic property of renin presumably because of unique enzyme-substrate interaction involving extensive portions of the interacting macromolecules. The possibility that it is due to contaminating protease such as cathepsin can be readily eliminated by the lack of protease activity and by the multiple criteria of purity.

Studies on the dependence of renin activity on substrate concentration revealed that renin activity reaches the maximal level at 50% of the plasma concentration (Table III). Therefore, renin in rat plasma is saturated by its substrate and change in the plasma substrate level has little effect on the rate of angiotensin generation in agreement with observations made by Johnston et al. [29] using impure plasma renin.

Species specificity of renin-angiotensinogen reaction is clearly demonstrated by data summarized in Table III. On the other hand, the difference between rat and hog renin disappears with the synthetic octapeptide substrate [14]. These findings indicate that the active site region of hog and rat renin shares common structural features, and that it is the interaction involving other areas of renin which contributes to the species specificity.

* After this paper had been submitted, a paper which included the purification of rat kidney renin appeared [30]. The purified rat renin obtained by these authors had a specific activity of 160 μg angiotensin I/μg per h using rat substrate, whereas our preparations tested under somewhat different conditions had 110 μg angiotensin I/μg per h. The difference between specific activities of the purest preparations of the two studies can very well be due to different methods of the determination of protein concentrations.

Acknowledgements

We are greatly indebted to Dr. Paul Fletcher for performing amino acid analyses, to Dr. Erwin Haas for his kind gift of standard rat renin, to Dr. Robert Neal for reading this manuscript and to Mr. Edward Price for his able technical assistance. This study was supported by U.S.P.H.S. grants HL-14192, HL-16114 and The American Heart Association Grant 76-1024.

References

1 Inagami, T. and Murakami, K. (1977) J. Biol. Chem. 252, 2978—2983
2 Cohen, S., Taylor, J.M., Murakami, K., Michelakis, A.M. and Inagami, T. (1972) Biochemistry 11, 4286—4293
3 Okamoto, K. and Aoki, K. (1963) Jap. Circ. J. 27, 282—293
4 Koletsky, S., Shook, R. and Rivera-Velez, J. (1970) Proc. Soc. Exp. Biol. Med. 134, 1187—1190
5 De Jong, W., Lovenberg, W. and Sjoerdsma, A. (1972) Proc. Soc. Exp. Biol. Med. 139, 1213—1216
6 Sen, S., Smeby, R.R. and Bumpus, T.M. (1972) Circ. Res. 31, 876—880
7 Devaux, C., Corval, P., Auzan, C., Ducloux, J. and Menard, J. (1973) C.R. Acad. Sci. (Paris) 277, 2561—2564
8 Lauritzen, M., Damsgaard, J.J., Rubin, I. and Lauritzen, E. (1976) Biochem. J. 155, 317—323
9 Rubin, I. (1972) Scand. J. Clin. Lab. Invest. 29, 51—58
10 Boyd, G.W. (1974) Circ. Res., 35, 426—438
11 Leckie, B. (1973) Clin. Sci. 44, 301—304
12 Day, R.P. and Luetscher, J.A. (1975) J. Clin. Endocrinol. Metab. 40, 1085—1093
13 Haber, E., Koerner, T., Page, L.B., Kliman, B. and Purnode, A. (1969) J. Clin. Endocrinol. Metab. 29, 1349—1355
14 Roth, M. and Reinharz, A. (1966) Helv. Chim. Acta 49, 1903—1907
15 Reinharz, A. and Roth, M. (1969) Eur. J. Biochem. 7, 334—339
16 Williams, H.R. and Lin, T. (1971) Biochim. Biophys. Acta 250, 603—607
17 Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751—766
18 Murakami, K. and Inagami, T. (1975) Biochem. Biophys. Res. Commun. 62, 757—763
19 Davis, B.J. (1965) Ann. N.Y. Acad. Sci. 121, 404—427
20 Andrews, P. (1964) Biochem. J. 91, 222—233
21 Shapiro, A.L., Vinuela, E. and Maizel, Jr., J.V. (1967) Biochem. Biophys. Res. Commun. 28, 815—820
22 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
23 Moore, S. and Stein, W.H. (1963) Methods Enzymol. 6, 819—831
24 Hirs, C.H.W. (1967) Methods Enzymol. 11, 50—62
25 Catley, B.J., Moore, S. and Stein, W.H. (1969) J. Biol. Chem. 244, 933—936
26 Vesterberg, O. (1972) Biochim. Biophys. Acta 257, 11—19
27 Inagami, T. and Murakami, K. (1977) Circ. Res. 41, Suppl. II, 11—16
28 Inagami, T., Murakami, K., Misono, K., Workman, R.J., Cohen, S. and Suketa, Y. (1977) in Acid Protease: Structure, Function and Biology (Tang, J., ed.), pp. 225—247, Plenum Publishing Co., New York
29 Johnston, C.I., Mathews, P.G., Davis, J.M. and Morgan, T. (1975) Pflüger's Arch. 356, 277—289
30 Hackenthal, E., Hackenthal, R. and Hilgenfeldt, U. (1978) Biochim. Biophys. Acta 522, 474—499