Application of Immunochemical Methods to the Identification and Characterization of Rat Kidney Inactive Renin

YUKIO TAKII, AMINTAS F.S. FIGUEIREDO, AND TADASHI INAGAMI

SUMMARY Identification of inactive prorenin in the kidney has been difficult due to rapid proteolytic conversion of the inactive zymogen to its active form in the tissue or during homogenization and purification. Immunochemical methods, Western blotting, direct radioimmunoassay, and immunofinity chromatography were used to isolate and identify rat kidney renin and prorenin and to determine their molecular weights without complete purification. Antisera to pure rat renin were raised in rabbits. A specific reaction between the antisera and rat renin was demonstrated by double immunodiffusion, inhibition of enzyme activity, and competitive radioimmunoassay. The anti-rat renin IgG did not cross-react with purified human renin or rat spleen or kidney cathepsin D. The IgG showed binding affinity to both inactive renin as well as active enzyme. A combination of affinity chromatographies consisting of pepstatin-Sepharose, IgG-Sepharose, and Affi-Gel Blue permitted rapid and complete separation of inactive renin from active renin in rat kidney extract. Neither inactive nor active renin preparations exhibited aspartyl protease activity on hemoglobin used as substrate. The apparent molecular weight of inactive renin was estimated as 50,000 by gel filtration. Electrophoresis of partially purified inactive renin in sodium dodecyl sulfate (SDS) polyacrylamide gel followed by transblotting of proteins to a nitrocellulose sheet and immunoochemical staining with anti-renin IgG showed a single protein band with a molecular weight of 48,000. Activation of inactive renin by trypsin was accompanied by the reduction of the 48,000-dalton native protein to a 39,000-dalton protein as determined by the SDS polyacrylamide gel electrophoresis and the transblotting. Gel filtration of active renin fraction in the kidney extract showed two forms of renin: high-molecular-weight (60,000) renin and low-molecular-weight (39,000) renin. High-molecular-weight renin was found to be a complex of low-molecular-weight renin and other protein(s) by SDS polyacrylamide gel electrophoresis analysis. Neither the high- nor the low-molecular-weight active renin showed a capacity for further activation by trypsin treatment. These results suggest that the inactive renin isolated in the present study is a zymogenlike precursor for renin and that it is different from active high-molecular-weight renin, which appears to be a complex of active enzyme and other proteins.

(Hypertension 7: 236–243, 1985)

KEY WORDS • rat renin antibodies • rat renal inactive prorenin • molecular weight • Western blotting • direct radioimmunoassay

SINCE the observation of spontaneous increase in renin activity of fresh renal extracts in acidic pH,1,2 similar or different types of the activatable renin or high-molecular-weight renin (molecular weight, 60,000) have been reported in the crude renin preparation from rabbit,3 hog,4 rat,5 dog,6 and human kidney7,8 and from Wilms' tumor.9 Most of these renins were already partially active, however, and their exact nature and identity were not clear unless the renin was at least partially purified.7,8 It was possible that some of them were complexes of active renin (molecular weight, 40,000) with other proteins of unknown nature.3,11–13 On the other hand, completely inactive renin can be either a proenzyme with a single polypeptide chain or a renin-inhibitor complex. Identification and isolation of inactive renin from the kidney for the purpose of its characterization have been hampered by its rapid spontaneous activation during its...
isolation because of high levels of proteases in kidney extracts. Studies on renal inactive renin require stabilization and rapid purification. Use of renin-specific IgG-Sepharose and other affinity columns permitted us to develop a partial purification method for inactive renin and to identify its molecular nature. Evidence was obtained that indicates the inactive renin in rat kidney is a single polypeptide protein of 48,000 daltons consistent with the structure of a proenzyme rather than a renin-inhibitor complex.

Materials and Methods

Pure renin was prepared by published methods from rat kidney, mouse submandibular gland, and human kidney. Cathepsin D was purified from rat spleen and rat kidney according to the published methods.

Preparation of Antibodies

Pure rat renin (500 μg) was conjugated with 250 μg of tetanus toxoid (40 μg, gift of Dr. F. Chytíl of Vanderbilt University, Nashville, TN) with 15 μg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Bio-Rad Laboratories, Richmond, CA) in an overnight reaction at 22°C and pH 5.0. The pH was maintained by the addition of 1 N HCl. Antisera were raised by immunizing Dutch-belted rabbits at multiple intradermal sites with the renin–tetanus toxoid complex (80 μg renin equivalent) in 50% Freund’s complete adjuvant (Miles Laboratories, Inc., Elkhart, IN) for the initial immunization followed by biweekly boosters with the same complex (10 μg renin equivalent for each booster) in 50% Freund’s incomplete adjuvant. The gamma globulin–containing fraction was precipitated from the pooled rabbit sera with 45% saturated ammonium sulfate, dialyzed against 0.01 M Tris-EDTA, 0.1% bovine serum albumin a full renin activity was retained for 16 hours at 4°C in the absence of antisera. The activity observed even with 200 μg of trypsin under these conditions. After reaction for 30 minutes at 22°C, trypsin was neutralized by the addition of 100 μg of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO).

Renin and Cathepsin Activity

Renin activity was determined by the amount of angiotensin I formed during the incubation of 25 μL of renin at 37°C with 100 μL of plasma of nephrectomized rats (0.9 μM) in 190 μL of 0.2 M MES (morpholineethanesulfonic acid) buffer, pH 6.1, containing 8.7 mM EDTA and 3.0 mM phenylmethylsulfonyl fluoride (PMSF) for 20 to 60 minutes. The angiotensin I formed was determined by the radioimmunoassay method of Haber and colleagues. Inhibition of renin activity by the anti-rat renin antiserum was tested by incubating 25 μL of renin with 25 μL of serially diluted antiserum for 16 hours at 4°C in the presence of 0.1% bovine serum albumin at pH 6.8. Aliquots (25 μL) of this mixture were used for assaying the remaining renin activity. In the presence of bovine serum albumin a full renin activity was retained for 16 hours at 4°C in the absence of antiserum. The activity observed without antiserum was defined as 100%.

Cathepsin D activity was determined with C-labeled hemoglobin used as a substrate at pH 4.5 and 37°C, according to the methods of Williams and Lin. Rat spleen cathepsin D and anti-cathepsin D IgG were prepared according to the methods of Yamamoto and co-workers.

Separation of Inactive Renin from Active Renin

The Pepstatin-Sepharose gel was prepared according to the published method. Rats were first stunned then decapitated. Whole frozen rat kidneys (950 g...
The adsorbed materials were eluted from the column with 4 M MgCl₂, the pH of which had been adjusted to 6.8, containing 1 mM EDTA according to the methods of Yokosawa and associates. It was washed with the phosphate buffer and eluted with stepwise increase of NaCl concentration to 0.5 M in the phosphate buffer.

Molecular Weight

Analytical gel filtration was run on a calibrated column (2.5 × 90 cm) of Sephadex G-100 (Pharmacia). In addition to the standard method of calibration, ³⁵S-labeled bovine serum albumin and ovalbumin were used as internal molecular weight standards. The sodium dodecylsulfate-polyacrylamide (SDS-PAGE) was run according to the methods of Laemmli. The molecular weights of immunoreactive substances were determined by Western blotting technique according to the methods of Towbin and co-workers, in which proteins were transferred electrophoretically by transverse electric current from an SDS gel (10-18% polyacrylamide) to a nitrocellulose sheet (Millipore Corp., Bedford, MA) that was layered over the SDS gel. The nitrocellulose sheet was then treated sequentially with anti-renin IgG (1:550, or anti-renin antiserum), goat anti-rabbit IgG (1:50, gift of Dr. R.J. Workman of Vanderbilt University) and rabbit anti-horseradish peroxidase–horseradish peroxidase complex (1:250; Cappel Laboratories, Downington, PA), then stained with 3,3′-diaminobenzidine in the presence of H₂O₂. Standard molecular weight proteins (molecular weight, 14,300–92,000; Bio-Rad) transferred to the nitrocellulose sheet were stained with 10% amido black.

The protein concentration was determined by the
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FIGURE 2. Displacement of $^{125}$I-renin from anti-renin IgG following exposure to rat renin or rat cathepsin D. Fifty microliters of anti-renin IgG diluted $1:100$ in Tris-bovine serum albumin (see text) was incubated with 50 $\mu$L of $^{125}$I-renin for 30 minutes at $4^\circ$C. The mixture was diluted with 850 $\mu$L of Tris-NaCl-EDTA-Triton-bovine serum albumin (see text) before the addition of various quantities of renin ($\odot$) or cathepsin D ($\Delta$). $B = antibody$-bound tracer; $B_0 = antibody$-bound tracer at zero base.

Folin-phenol reagent with bovine serum albumin (Sigma) used as a reference.26

Results

Immunological Studies

Double diffusion of pure rat renin (electrophoretically homogenous) or crude rat renin preparations against anti-rat renin antisera on an agar plate produced a single precipitin line. A faint line was formed with hog kidney renin. No precipitin reaction was produced with human or mouse submandibular gland renin. On the other hand, the anti-rat renin antisera exhibited the ability to inhibit the enzyme activity of renin obtained from rat and hog kidneys and mouse submandibular gland (an example is shown in Figure 1). The antisera did not affect the human kidney renin activity even at the highest concentration tested ($1:38$ dilution). Similar inhibitory activities were observed with crude IgG. The antisera ($1:100$ dilution) did not inhibit cathepsin D obtained from rat spleen17 or rat kidney.18 Tested by immunohistochemical staining of rat kidney sections, the antisera stained juxtaglomerular cells exclusively sparing all other cells.27 The affinity of pure rat spleen cathepsin D to the anti-renin antiserum or IgG was tested with the direct radioimmunoassay method for renin (Figure 2). By this method approximately $100$ pg of renin can be detected. Dissociation of bound $^{125}$I-renin was not observed even in the presence of an excess ($10$ ng) of cathepsin D. Anti-cathepsin D IgG17 ($1:10$ dilution) did not affect the activity of pure rat kidney renin.

Separation of Inactive Renin and Active Renin

As shown in Figure 3, renin activity was eluted from pepstatin-Sepharose in two separate peaks. The first renin peak in the pass-through fractions contained only a small portion ($0.5\%$) of the total renin activity (open circles). The renin activity in these fractions increased markedly after the trypsin treatment (closed circles). The second renin peak in Figure 3 eluted by $0.1$ M Tris-hydrochloride buffer accounted for approximately $80\%$ of the total renin activity applied to the column. The trypsin treatment of the second fractions resulted in a slight reduction in the enzyme activity, not a further increase. The first renin peak in Figure 3 was adsorbed to the anti-rat renin IgG-Sepharose column and eluted by $4$ M MgCl$_2$ (Figure 4). Active renin

FIGURE 3. Affinity chromatography of rat kidney extract on a pepstatin-Sepharose gel. The column was eluted with $1.0$ M NaCl in $0.02$ M phosphate buffer, pH $6.5$ (first arrow), which was followed by $0.1$ M Tris-hydrochloride buffer, pH $7.5$ (second arrow). Renin activity ($\odot$) was determined before ($\odot$) and after ($\bullet$) activation by trypsin. ($\cdots$) = absorbance at $280$ nm; angio = angiotensin I

FIGURE 4. Immunoaffinity column chromatography of pooled inactive renin fractions obtained from the pepstatin-gel (the second activity peak in Figure 3). The first arrow indicates the start of washing with $0.1$ M HEPES buffer, pH $7.1$, containing $1$ mM EDTA and $10\%$ ethylene glycol. The second arrow indicates the start of elution with $4$ M MgCl$_2$ solution. $\odot = renin$ activity ($\cdots$) before trypsin activation; $\bullet = renin$ activity after trypsin activation; ($\cdots$) = absorbance at $280$ nm; angio = angiotensin I; the bracket indicates fractions used for the next step of purification.
Molecular Weight of Inactive Renin and Active Renin

The molecular weight of inactive renin was estimated by gel filtration on a Sephadex G-100 column. The inactive renin was eluted in fractions corresponding to a molecular weight of 50,000 (Figure 6B). (The recovery of the potential enzyme activity from the Sephadex column was greater than 75%.) On the other hand, gel filtration analysis of active renin revealed an elution pattern of two separate peaks corresponding to the molecular weight of 60,000 and 40,000 respectively (Figure 6A), as estimated in reference to internal molecular weight standards (14C-labeled bovine serum albumin and ovalbumin). The SDS-PAGE of inactive renin in the presence of 2-mercaptoethanol followed by Western blotting showed a single immunoreactive renin band with a molecular weight of 48,000 (Figure 7, Lane 3). On activation of inactive renin by trypsin,

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\text{(Figure 4, open circles), which was co-eluted in the trypsin-activatable renin fractions (closed circles), was completely removed by a passage through an Affi-Gel Blue column, as shown in Figure 5. Fractions that were eluted with 0.5 M NaCl from this column did not show renin activity beyond the control level (Figure 5), but the trypsin treatment of the fractions resulted in extensive activation, which indicates the presence of a completely inactive renin in these fractions. These fractions were pooled and used as inactive renin in subsequent studies. Active renin obtained from the pepstatin-Sepharose column (second renin peak in Figure 3) was applied to the anti-renin IgG-Sepharose column to obtain a further purified active renin (not shown) and used as active renin. The results of the purification of inactive renin from the kidney are summarized in Table 1. The IgG-Sepharose chromatography was the most important step in this partial purification procedure, which accomplished a 900-fold purification in a single step. The final product of this partial purification work was estimated to be approximately 7% pure by comparing its potential specific activity with that of pure rat renin.}
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\text{TABLE 1. Partial Purification of Inactive Renin from Rat Kidney*}
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<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
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<th>Yield (%)</th>
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<tr>
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<td>0.110</td>
<td>8300</td>
<td>24,200</td>
<td>6.7</td>
</tr>
</tbody>
</table>

*Starting from 950 g of rat kidneys.  
†Per microgram of angiotensin I per hour per milligram of protein.  
‡Diethylaminoethyl cellulose.

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\text{Figure 6. Sephadex G-100 chromatography of active and inactive renin. A. Active renin obtained from the pepstatin-gel-bound fractions (second renin peak in Figure 4) followed by IgG-Sepharose. B. Inactive renin obtained from the Affi-Gel Blue column (under the bracket in Figure 5). ○ = renin activity before activation by trypsin; ● = renin activity after activation; angio = angiotensin I; } V_e = \text{ elution volume; } V_o = \text{ void volume. Arrows indicate the elution positions of the internal molecular weight standards. 14C-labeled bovine serum albumin and ovalbumin.}
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FIGURE 7. The SDS-PAGE of inactive renin and active renin. Aliquots of the sample solution were applied to slabs consisting of 10 to 18% gradient polyacrylamide gel. The electrophoresis was carried out according to the methods of Laemmli. The proteins in each gel track were transferred to nitrocellulose sheet according to the methods of Towbin and colleagues. The tracking dye was bromophenol blue. Standard molecular weight proteins were stained with amido black. The other electrophoretograms were treated sequentially with rabbit anti-rat renin IgG, goat anti-rabbit IgG, rabbit anti-horseradish peroxidase–horseradish peroxidase complex, 3,3′-diaminobenzidine, and H2O2. Lane 1: standard molecular weight proteins (58,000, 40,000, and 31,000); Lane 2: standard molecular weight proteins (92,000, 67,000, 46,000, 31,000, 21,000, and 14,000); Lane 3: inactive renin (150 pg); Lane 4: active renin produced from inactive renin (150 pg) by trypsin treatment; Lane 5: active low-molecular-weight-renin (120 ng from Figure 6A); Lane 6: active high-molecular-weight renin (100 ng from Figure 6A); Lane 7: pass-through fractions from pepstatin-Sepharose (100 ng; Figure 4); Lane 8: renin fractions eluted from pepstatin-Sepharose (100 ng; bracket in Figure 4); Lane 9: purified rat renin (1.4 μg); Lane 10 as for Lane 9, but with normal rabbit serum used as the primary antibody. Renin concentration in the sample was determined by direct radioimmunoassay before the electrophoresis.

Discussion

Due to exceedingly small quantities and instability of renin and inactive renin, studies on their molecular properties have encountered great difficulties. Characteristic selectivity and high affinity of specific antibodies seem to make immunochemical methods attractive tools to cope with these problems. In the present studies we have devised several immunochemical approaches for the characterization of inactive renin in rat kidney, which has long eluded conventional biochemical approaches. Usefulness of antibodies for the identification of renin has been amply demonstrated. The appearance of inactive renin in the plasma of totally nephrectomized human subjects suggested possible extrarenal sources of inactive renin; however, recent studies have demonstrated the presence of inactive renin in human kidney. The absence of inactive renin in kidneys of various nonprimate mammals gave the impression that these species do not store inactive renin in the kidney. Recent identification of inactive renin in hog kidney by Takii and Inagami, however, suggests the synthesis and storage of inactive renin in the kidney of this species. Identification of inactive renin in rat kidney by its isolation has been even more difficult. Results of the present study indicate the presence of inactive renin in the rat kidney. This finding is compatible with the observation of inactive renin in rat plasma by Barrett and co-workers and suggests the kidney as a source of inactive renin in plasma. Difficulties encountered in the identification of inactive renin in the kidney seem to be partially caused by the activation of inactive renin by a variety of proteases present at high concentrations in the kidney. The application of immunoaffinity chromatography permitted isolation and stabilization of inactive renin.

Whether the inactive renin is a simple polypeptide precursor of a zymogen type or a renin-inhibitor complex has been difficult to determine. It was only after...
the complete purification of inactive renin from hog kidney that definitive evidence for the single polypeptide zymogen was obtained.\textsuperscript{50} In view of the minute quantity and rapid activation by proteases, such a purification was exceedingly difficult. In the present study the inactive renin prepared by affinity chromatographic steps was by no means homogeneous; however, use of specific antibodies to renin in the Western blotting method in combination with SDS-PAGE permitted us to demonstrate that inactive renin in rat kidney is a protein consisting of a single polypeptide chain with a molecular weight of 48,000 to 50,000.

Although the kidney seems to contain two species of active renin with molecular weights of 40,000 and 60,000, results of the SDS-PAGE reduced the molecular weight of the larger species to a lower value indistinguishable from the 37,000- to 40,000-dalton active renin. The 60,000- to 50,000-dalton active renin seems to be a complex of the 40,000-dalton renin and a binding protein — as suggested by Inagami and Murakami\textsuperscript{11} and Funakawa and colleagues\textsuperscript{12} for pig and dog kidneys respectively — because it was prepared in the presence of sodium tetrathionate under the condition that was shown to form renin-binding protein complex.\textsuperscript{11}

These observations support the concept that the inactive 48,000- to 50,000-dalton renin is a zymogen and is converted to 37,000- to 40,000-dalton active renin by limited proteolysis. Active renin purified from rat kidneys was shown to have a molecular weight in the vicinity of 37,000 to 40,000\textsuperscript{13,17} which is in agreement with the 37,000- to 40,000-dalton band of the active renin obtained in the present studies.

The molecular weight of rat kidney inactive renin (48,000–50,000) estimated by SDS-PAGE and Western blot is in general agreement with the molecular weight (50,000) of the translation product of the mouse kidney renin messenger ribonucleic acid.\textsuperscript{32,33} The size is somewhat larger than the prorenin derived from mouse submandibular gland messenger ribonucleic acid (45,000).\textsuperscript{32,34} One of the reasons for the difference may be due to the absence of carbohydrates in the submandibular gland enzyme or in the in vitro translate of messenger ribonucleic acid.

With the currently available antibodies, 100 pg of rat renin is detectable by the direct radioimmunoassay method. As the antibodies used in this study specifically react with renin of the rat, hog, and mouse in exclusion of human renin or rat cathepsin D, the antibody should be useful in distinguishing the angiotensin-forming activity of renin in tissue extracts from the similar but nonspecific activity of cathepsin D, which is present in large quantities in many organs. The specificity of this antibody was also suggested by exclusive immunohistochemical staining of juxtaglomerular cells with all other cells remaining intact in rat kidney sections. The anti-renin antibody does not appear to distinguish active renin from inactive zymogen.

\textbf{Addendum}

While this manuscript was in the process of being reviewed Pratt and coworkers\textsuperscript{60} and Catanzarro and associates\textsuperscript{70} published reports that support the thesis that a single polypeptide renin precursor produces renin by limited proteolysis and thus can be considered as renin zymogen.

\textbf{Acknowledgment}

The authors are greatly indebted to Mr. E. Price, Jr., for his skilful assistance, to Dr. R. J. Workman for a generous gift of goat anti-rabbit IgG, and to Dr. F. Chytil for his kind gift of pure tetanus toxoid.

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Y Takii, A F Figueiredo and T Inagami

Hypertension. 1985;7:236-243
doi: 10.1161/01.HYP.7.2.236

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1985 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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