Isolation and Activation of Inactive Renin from Human Kidney and Plasma

Plasma and Renal Inactive Renins Have Different Molecular Weights

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SUMMARY Inactive renin and active renin from human kidney and human plasma were prepared in highly purified forms by three steps of chromatography on Octyl-Sepharose, immunoaffinity chromatography, and pepstatin-amino hexyl Sepharose CL-4B. The inactive renin and active renin from human kidney had molecular weights of 51,000 and 44,000 as measured by a calibrated gel filtration column run with internal molecular weight standards. Molecular weights of plasma inactive renin and active renin were 56,000 and 51,000 respectively. Both inactive and active renins were found to be heterogeneous, consisting of several components with different isoelectric points. Renal inactive renin has higher pi values of 6.40, 6.10, 5.90, 5.61, and 5.40. Renal active renin has pi values of 5.73, 5.40, 5.25, and 5.13. The pi values of plasma inactive renin were 6.37, 6.08, 5.77, 5.36, and 5.25; of plasma active renin, 5.68, 5.40, 5.33, and 5.25. Trypsin activation and plasmin activation of plasma inactive renin produced an active enzyme with similar molecular weight but lower pi values. Acid activation of inactive renin did not change the molecular weight and pi values.

KEY WORDS • inactive renin • human kidney • human plasma • acid activation • plasmin activation • molecular weight • isoelectric point • affinity chromatography • octyl-Sepharose immunoaffinity chromatography

Since the ratio of active to inactive renin in human plasma varies under different physiopathological conditions, it is likely that the conversion of inactive renin to active renin plays a role in the regulation of plasma renin activity (PRA). Whether such activation takes place in the plasma or in the kidney has not been clarified. Since the major source of the circulating renin is in the kidney, the possibility that the inactive renin is synthesized and activated in the kidney cannot be excluded. In spite of numerous studies on inactive renin in crude renal extracts from the kidneys of rats, hog, dog, and rabbit, the nature of renal inactive renin has remained elusive. Several attempts to isolate such inactive renin have produced a large size renin that is already active. To date, most of the activatable forms of renal renin have been considered as complexes of active renin and other proteins.

The present studies were initiated to determine if totally inactive renin exists in human kidney and to see if the renal inactive renin is different from plasma inactive renin. Studies on renal inactive renin require stabilization by rapid and partial purification. We have developed a highly efficient purification method and applied it to both human renal and plasma inactive renin. A totally inactive renin was prepared both from human plasma and kidney. Using these inactive renin preparations, we determined the molecular characteristics. We observed intriguing discrepancies between the molecular weights of renal and plasma inactive renins. A preliminary report of these studies has been published in abstract form.

Materials and Methods

Human kidneys obtained by autopsy or surgery were stored frozen until use. Three times recrystallized trypsin, soybean trypsin inhibitor, crystalline bovine serum albumin, ovalbumin and lysozyme, protease inhibitors, diisopropyl fluoro-phosphate (DFP), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Company. Human plasmin was obtained from Kabu A. G. of Sweden. Octyl-Sepharose CL-4B was from Pharmacia Fine Chemicals. N,N-bis-(2-hydroxy-ethyl)-2-amino-ethanesulfonic acid (BES) and 2-(N-morpholino)-ethanesulfonic acid (MES) were from Calbiochem-Behring Corporation. Triton X-100 was from Research Product International Corporation. All other reagents were of reagent grade.

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Purification of Inactive Renin from Human Kidney

Frozen human kidneys were thawed partially. The cortex was separated from the medulla and homogenized at 4°C in 0.1 M BES-NaOH buffer, pH 7.15, containing the following protease inhibitors: EDTA (5 mM), PMSF (1 mM), DFP (1 mM), leupeptin (5 mg/l), and aprotinin (10 mg/l). The homogenate was centrifuged at 5000 rpm for 1 hour. Fine powder of ammonium sulfate was added slowly to a clear supernatant, and proteins precipitated between 25% and 60% saturation were collected by centrifugation. The pellet was redissolved in 10 mM phosphate buffer, pH 6.8, containing 1 M ammonium sulfate, 2 mM EDTA, and 1 mM PMSF. The solution was then dialyzed overnight against the same buffer, with three changes of the buffer.

After removal of insoluble materials by centrifugation for 30 minutes at 15,000 rpm, the supernatant was subjected to hydrophobic interaction chromatography on an Octyl-Sepharose CL-4B column (6 × 30 cm), which had been pretreated with 1% Triton X-100 and equilibrated with the phosphate buffer containing ammonium sulfate, EDTA, and PMSF, as mentioned above. After the application of the supernatant, the column was washed with 1.5 liters of the same buffer. The adsorbed proteins were eluted with a decreasing ammonium sulfate concentration gradient. Fractions with inactive renin (fig. 1) were pooled and concentrated by pressure filtration to 25 ml using an Amicon PM 10 filter.

Inactive renin solution thus obtained was applied to an immunoaffinity column19 (1.5 × 5 cm) containing Sepharose coupled to rabbit antihuman renal renin IgG, which had been equilibrated with 50 mM Tris-HCl buffer, pH 6.8 containing 0.3 M KCl. After the application of the sample, the column was washed with 50 ml of the same buffer containing 0.1% Triton X-100 and 3 M urea to remove nonspecifically bound proteins; this was followed by an additional wash with 50 ml of the original buffer without the detergent and urea. The absorbed materials, which included inactive renin and small amount of active renin, were eluted with 4 M MgCl₂ solution adjusted to pH 6.0 with Tris-base (fig. 2). Fractions containing both active renin and inactive renin were pooled, and lysozyme was added to a concentration of 0.1% to protect the dilute inactive renin solution from absorptive loss. After dialysis against two changes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl, then against three changes of 50 mM pyrophosphate buffer, pH 6.0, the solution was applied to a pepstatin-amino-oxycarbonyl-Sepharose CL-4B column (1.5 × 5 cm), which had been washed with 8 M urea and equilibrated with 50 mM pyrophosphate buffer, pH 6.0, containing 0.1% lysozyme. While active renin was bound to the column, inactive renin appeared in the pass-through fractions. Active renin absorbed in the pepstatin column was eluted with 50 mM Tris-HCl buffer, pH 7.4 (fig. 3). Active renin fractions obtained from the Octyl-Sepharose column was treated in the same way to obtain a highly purified active renal renin from human kidney.

Human plasma inactive renin was purified in a similar manner from freshly frozen blood bank plasma by Okamoto's modification (to be published) of the method of Yokosawa et al.17 In brief, the plasma (800 ml) was thawed in the presence of DFP (added to a final concentration of 10 mM), centrifuged to remove insoluble materials, and fractionated with ammonium sulfate. A fraction precipitated between 30% and 60% saturation of ammonium sulfate was used as starting material and purified in a manner similar to that of the renal inactive renin. This precipitate containing proteins and ammonium sulfate was dissolved in a small volume of 10 mM sodium phosphate buffer, pH 6.8, containing 2 mM EDTA.
and 0.5 mM DFP, then was purified successively on columns of Octyl-Sepharose, anti-renin-IgG-Sepharose, and pepstatin-Sepharose, by the method described above. The final product was stabilized in 0.1% hen egg white lysozyme.

Renin Activity Determinations

Renin activity was determined by radioimmunoassay* of angiotensin I generated in 1 hour from partially purified human angiotensinogen at 37°C in a mixture made up with 190 µl of MES buffer pH 6.0, 10 µl of 0.2 M EDTA, 5 µl of PMSF (25 mg/ml of isopropanol), 25 µl of the angiotensinogen solution (77 µg angiotensin I equivalent), and 120 µl of an appropriately diluted renin sample.

Activation of Inactive Renin by Proteases

Solution containing renal inactive renin (10 µl) or plasma inactive renin (50 µl) was mixed with 40 µl of 1 M Tris-HCl, pH 7.5. The total volume was made to 90 µl with an additional volume of distilled water. The activation reaction was started by adding 10 µl (5 µg) of a trypsin-BSA solution which was prepared by dissolving 5 µg of trypsin in 1 ml of 1 mN HCl then diluting it with 9 volumes of 5% BSA. After 10 minutes at 22°C, the reaction was stopped by the addition of 10 µl of soybean trypsin inhibitor solution (5 mg/ml) in 0.2 M MES buffer, pH 6.0. Activation by plasmin was performed similarly with 5 µg of plasmin in 50% glycerol for 37°C for 30 minutes.

Acid Activation

The inactive renin solution (0.5 ml) was dialyzed against 50 mM glycine-HCl buffer, pH 3.2, containing 0.1 M NaCl at 4°C for 20 hours. The activity of the acid-activated solution (20 µl) was measured by incubating it with the angiotensin I-generating mixture (330 µl) at pH 6.0. To reverse the activation, the acid-treated solution was dialyzed against Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl at 4°C overnight, and pH was readjusted to 6.0 for activity assay.
Molecular Weight Determinations

The molecular weights of renins were determined by gel filtration on a calibrated Sephadex G-100 superfine column (1.5 × 90 cm) which was eluted with 20 mM Na-pyrophosphate, pH 7.1, containing 0.9% NaCl, 0.1% lysozyme, and 0.2% sodium azide as elution buffer. BSA, ovalbumin, and soybean trypsin inhibitor, labeled with [14C] formaldehyde by reductive alkylation with sodium cyanoborohydride in 6 M guanidine, were added to sample solutions as internal molecular weight standards. The radioactivity was measured by scintillation counting. Addition of microgram quantities of these internal standards did not perturb the elution pattern of plasma or kidney proteins. The elution positions of active renin and inactive renin were determined by radioimmunoassay of renin activity before and after trypsin activation of each fraction.

Isoelectric Focusing

Isoelectric points were determined by electrofocusing renin-containing solutions in 5% polyacrylamide gels with 2.5% cross-linkage containing 5% ampholine. Gel strips 10 cm long were cut to 2.5 mm slices and extracted overnight either with distilled water or with 50 mM Tris-HCl buffer, pH 7.4, containing 0.5% NaCl and 0.1% BSA. The pH of the extract was determined by glass electrodes, and the renin activity was determined as described above.

Results

Separation of Inactive Renin and Active Renin

As shown in figure 1, inactive renin and active renin in human kidney extract were separated by the hydrophobic chromatography on Octyl-Sepharose previously washed and regenerated with the detergent Triton X-100. Since the inactive renin tended to undergo spontaneous activation even after this first step, presumably by contaminating proteases, it was essential to add protease inhibitors to preserve the inactive form or to suppress its conversion to active form. The 3-step affinity chromatographic procedure was completed by chromatography on a pepstatin amino-addy-Sepharose, which removed the active enzyme generated during the isolation procedure and produced completely inactive renin. Chromatography on Octyl-Sepharose resulted in approximately 15-fold purification for kidney inactive renin, and 40-fold purification for plasma inactive renin. The immunoadfinity chromatography contributed greatly to purification, resulting in 30-fold purification for kidney inactive renin and 160-fold purification for plasma inactive renin. Essentially similar results were obtained with autopsied kidneys and surgical kidneys. The extent of the overall purification could not be estimated since the content of inactive renin in crude extracts was not determined reliably due to rapid activation. The product of the purification was probably not completely pure but did not undergo spontaneous activation. Upon trypsin activation, plasma inactive renin showed specific activities between 14 and 20 GU/mg whereas renal inactive renin had specific activities between 41 and 55 GU/mg. The active renin used for comparison was also purified by the immunoaffinity column.

Activation

Totally inactive renin was obtained from both human plasma and human kidney. The renal inactive renin was activated to the maximal level by both trypsin and plasmin, whereas plasma inactive renin was activated more extensively by plasmin than trypsin (table 1). This difference may be due to difference in their molecular size and hence difference in some molecular properties.

<table>
<thead>
<tr>
<th>Conditions for Activation</th>
<th>Kidney</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity (ng Al/ml)</td>
<td>Activation* (%)</td>
</tr>
<tr>
<td></td>
<td>(n = 3)</td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>0 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>33.3 ± 1.0</td>
<td>100</td>
</tr>
<tr>
<td>Plasmin</td>
<td>32.7 ± 1.0</td>
<td>98</td>
</tr>
<tr>
<td>Acid activation steps:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 3.2†</td>
<td>30.6 ± 1.0</td>
<td>96</td>
</tr>
<tr>
<td>pH 3.2-pH 7.5‡</td>
<td>5.6 ± 0.5</td>
<td>18</td>
</tr>
<tr>
<td>pH 3.2-pH 7.5-pH 3.2§</td>
<td>36.7 ± 0.5</td>
<td>110</td>
</tr>
</tbody>
</table>

*Percentage activation was calculated in reference to the highest extent of activation either by trypsin or plasmin.
†Assayed immediately after treatment at pH 3.2 for 20 hours in the cold. Values are mean ± standard deviation (SD).
‡After pH 3.2 treatment, it was treated at pH 7.5 for 20 hours.
§The sample c was treated again at pH 3.2 for 20 hours.
The acid activation obtained by dialysis against an acid buffer of pH 3.2 for 20 hours resulted in complete activation if the activity was determined at pH 6.0 immediately after the acid treatment. However, upon subsequent treatment at pH 7.5, the gain was almost completely lost. Repeated treatment at pH 3.2 again reproduced the activation analogous to the reversible activation of inactive renin in human plasma observed by Hsueh et al.4 and Leckie5 (table 1).

Molecular Weight

The apparent molecular weight of the inactive renin as determined by a calibrated Sephadex G-100 column was 51,000 ± 1,500 (SD, n = 4) for the renal enzyme, and 56,000 ± 1,500 (SD n = 4) for the plasma enzyme. Upon activation by plasmin, little changes in the apparent molecular weight were observed (table 2). Activation by trypsin caused some reduction in molecular weight of the renal enzyme but not that of plasma enzyme. In both cases, the maximum enzyme activity reached by the trypsin treatment was less than optimal. The enzyme isolated in the active form from the renal cortex showed a molecular weight discretely lower than proenzyme (p < 0.01). This finding was further confirmed by the observation that it was lower than that of 14C-labeled ovalbumin used as internal molecular weight standard.

Inactive renin isolated in a similar manner from freshly frozen human plasma of normal blood donors had a definitely higher molecular weight of 56,000 than renal inactive renin (p < 0.01) (table 2). Active renin from human plasma was also larger than that of active renin from the kidney (p < 0.01). Activation of inactive renin with various proteases did not reduce the molecular weight appreciably.

Isoelectric Point

Isoelectric point was also used as an additional index of molecular properties for the comparison of active and inactive renin. Multiple bands with renin activity were obtained upon isoelectric focusing on ampholine-containing polyacrylamide gel plates. The isoelectric points (pI) were estimated from the peak positions of the multiple bands exhibiting enzyme activity. Peaks for inactive renin were determined after activation with trypsin (figs. 4 and 5).

Both inactive renin and active renin in the kidney and plasma consists of multiple components that can be segregated by isoelectric focusing. Interestingly, the focusing patterns of inactive renins in the kidney and plasma are quite analogous to each other; they consist of five peaks each of similar pI values, as shown in figure 4 A (kidney inactive renin) and figure 5 A (plasma inactive renin).

Appreciable downward shifts occurred upon activation by plasmin or trypsin. Thus, kidney inactive renin peaks with pI values of 6.40, 6.10, 5.90, 5.61, and 5.40 (SD ≤ 0.04, n = 3) were shifted to 5.73, 5.54, 5.30, 5.20, and 5.00 (SD ≤ 0.03, n = 3) by plasmin activation, as shown in figure 4 B. Trypsin activation caused similar but not identical shifts (fig. 4 C). Renin isolated in an active form also had lower pI values of 5.73, 5.40, 5.25, and 5.13 (SD ≤ 0.03, n = 3). Similar downward shifts were observed with plasma renin. Activation with plasmin shifted five plasma proenzyme peaks with pI values of 6.37, 6.08, 5.77, 5.36, and 5.25 (SD ≤ 0.03, n = 3) to three peaks of active renin with pI values of 5.50, 5.36, and 5.30 (SD ≤ 0.03, n = 3).

TABLE 2. Molecular Weights of Human Renin

<table>
<thead>
<tr>
<th>Renin</th>
<th>Kidney (n = 4)</th>
<th>Plasma (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactive</td>
<td>51,000 ± 1,500</td>
<td>56,000 ± 1,500</td>
</tr>
<tr>
<td>Acid-treated*</td>
<td>51,000 ± 1,400</td>
<td>56,000 ± 1,300</td>
</tr>
<tr>
<td>Activated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin-treated</td>
<td>47,000 ± 3,000</td>
<td>56,000 ± 1,600</td>
</tr>
<tr>
<td>Plasmin-treated</td>
<td>51,000 ± 1,300</td>
<td>56,000 ± 1,500</td>
</tr>
<tr>
<td>Active</td>
<td>45,000 ± 1,200</td>
<td>51,000 ± 1,400</td>
</tr>
</tbody>
</table>

*Acid-treated first by dialyzing against pH 3.2 buffer overnight and then against pH 7.5 buffer overnight. Values are mean ± standard deviation (SD).

FIGURE 4. Isoelectric focusing of human renal inactive and active renin. A. Inactive renin. B. After plasmin activation. C. After trypsin activation. D. After acid activation. E. Active renin directly isolated from the kidney. Closed circles indicate activity measured without activation; open circles represent activity determined after trypsin activation of inactive renin solutions eluted from gel slices 2.5 mm wide.
Trypsin activation resulted in similar shifts. Active plasma renin also showed lower pi values of 5.68, 5.40, 5.33, and 5.25 (SD ≤ 0.04, n = 3).

Noteworthy is the absence of such downward shifts upon acid activation. The fully acid-activated enzyme was applied in the region of acid pH of the previously electrofocused slab gel immediately after the activation. Resultant focusing patterns of the kidney enzyme (fig. 4 D) and plasma enzyme (fig. 5 D) were almost identical with their respective precursors (fig. 4 A and fig. 5 A). Reversible inactivation and repeated activation at pH 3.2 did not change these electrofocusing patterns.

Discussion

The present study clearly indicated the existence of completely inactive renin in human kidney analogous to that in hog kidney. It also showed that the inactive renin can be isolated for the purpose of characterization. The characterization of human renal inactive renin has been extremely difficult due to its rapid and spontaneous activation in crude renal extracts. The rapid separation from active renin and bulk of other proteins by Octyl-Sepharose and subsequent extensive purification by immunoaffinity column no doubt contributed to the stabilization of inactive renin through the removal of much of proteases of renal origin. However, even by this method it seemed that a large portion of inactive renin had been converted to the active form before the activation was arrested completely. We and other investigators employed Affi-Gel Blue for the separation of plasma and renal inactive renins. The hydrophobic chromatography on Octyl-Sepharose produced equally reproducible or better results when applied to plasma and renal cortical extracts. A useful feature of this gel is that it permits rapid processing of ammonium-sulfate-precipitated proteins since the precipitated protein can be applied to this column simply by dissolving it in 1 M ammonium sulfate without desalting.

Inactive and active renin in plasma have molecular weights appreciably greater than their respective renal counterparts, as shown earlier by Yokosawa et al. and by the present studies. Since practically all of plasma renin seems to derive from the renal source, these observations suggest three alternative possibilities. First, active renin and inactive renin from the kidney may have been partially degraded during homogenization, extraction, and chromatography. Second, it is also possible that the smaller renal renin may have acquired a binding protein to form a larger counterpart in plasma either during the process of secretion or in plasma. A third possibility may be that renal inactive renin undergoes partial unfolding upon secretion, which may cause increase in its apparent molecular weight due to increase in Stokes' radius. The first possibility seems to be supported by direct radioimmunoassay of renin fractionated by SDS-gel electrophoresis immediately following rapid arrest of its proteolytic degradation by direct treatment of the tissue with hot SDS solution at 95°C. On the other hand, the existence of binding proteins in renin-producing tissues has been recognized. To distinguish these problems, further studies are needed using new techniques that can cope with the lability of inactive renin in the kidney.

The isoelectric focusing studies clearly show that both renal and plasma inactive renin consist of no less than four components. Upon protease-mediated activation, pi values of these inactive renin components undergo drastic downward shifts, suggesting loss of an appreciable number of basic amino acid residues during the activation.

The present studies also show that renal inactive renin undergoes reversible and complete activation by acid treatment. Analogous to similar reversible activation of plasma prorenin, the complete activation of renal inactive renin is detected only if the activity is determined immediately after the acid treatment at pH below neutrality. Apparently, the activated form is frozen at pH at or below 6.0, whereas at pH 7.5 it rapidly reverts back to the inactive form. The reversi-
ble nature and the absence of shift in pI values of the
cacid activation strongly indicates that the acid activa-
tion involves a conformational change in contrast to
protease activation, which involves limited proteolysis
of inactive renins and is irreversible. These obser-
vations are in support of those made by Hsueh et al. and Leckie
using plasma prorenin.

Active human renin was purified in different forms. Yokosawa et al. have purified a 40,000 dalton form of renin from acid extracts of human kidney. Galen et al. have purified renin from renin-secreting tumors in 50,000 dalton forms, which was shown to be a mixture of 50,000 dalton complexes and simpler peptides. Slater et al. have obtained a similar preparation from autopsied kidney (personal communication). It is likely that a high molecular weight material was degraded to a smaller molecule during extraction and chromatography. The possibility for such degradation is suggested by the reduction of the molecular weight of inactive renin by trypsin as experienced in the present study (p < 0.05). However, activation with plasmin did not alter the molecular weight of the activated product appreciably from that of inactive renin, suggesting the possibility that 50,000 dalton active renin may occur under natural conditions. The 44,000 dalton active renin found in the kidney extract may represent a further degraded form.

Addendum

After this manuscript had been submitted, similar studies on the separation of inactive and active renin in human kidney were published by Atlas et al.

References

Isolation and activation of inactive renin from human kidney and plasma. Plasma and renal inactive renins have different molecular weights.
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