Role of Glandular Kallikrein in the Activation Process of Human Plasma Inactive Renin

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SUMMARY Completely inactive renin was isolated from normal human plasma by DEAE-Sepharose column chromatography and Blue-Sepharose column chromatography. This inactive renin had a molecular weight of 54,000 daltons as determined by gel filtration on Ultrogel AcA 44. When the inactive renin was activated by trypsin, its molecular weight decreased to 48,000 daltons. The trypsin-activated renin differed from a native form of active renin in plasma with respect to molecular weight (active renin, 43,000), pi value (active renin, 5.20; trypsin-activated renin, 5.06), km value (active renin, 60 nmoles/liter; trypsin-activated renin, 89 nmoles/liter), Ki value for pepstatin A (active renin, 2.6 μmoles/liter; trypsin-activated renin 5.0 μmoles/liter) and pH profile for angiotensin formation. Glandular kallikrein (human urinary or pig pancreatic) did not activate the inactive renin. When the trypsin-activated renin was treated with glandular kallikrein, its activity was unchanged, but its molecular and kinetic properties except pi value (trypsin-activated kallikrein-treated renin, 4.82) coincided with those of a native form of active renin in plasma. These results indicate that glandular kallikrein does not directly activate inactive renin but participates in the activation process of inactive renin. The results also suggest that inactive renin in human plasma is a renin precursor.

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KEY WORDS • inactive renin • active renin • trypsin activation • glandular kallikrein • molecular weight

A n inactive renin ("prorenin") is present in human plasma. This plasma inactive renin has been shown to cross-react with the specific antibody to human renal renin. The in vivo activation process of the inactive renin in human plasma and its clinical significance are of great current interest. The inactive renin is activated in vitro by limited proteolysis using trypsin, pepsin, plasma and glandular kallikrein, and other proteolytic enzymes such as plasmin, fibronolysin, and cathepsin D. Sealey et al. reported that human urinary kallikrein converted the inactive renin to the active form. Yokozawa et al. and we have shown that the inactive renin that was separated completely from active renin was not activated by glandular kallikrein. After acid treatment, however, the inactive renin was activated by kallikrein, which suggested that the inactive renin required a structural alteration prior to activation by glandular kallikrein.

In this paper, we studied the role of human or pig glandular kallikrein in the activation process of human plasma inactive renin that was separated completely from active renin.

Materials and Methods

DEAE-Sepharose CL-6B and Blue-Sepharose CL-6B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Ultrogel AcA 44 and Ampholine pH 4-6 and pH 6-8 were from LKB, Bromma, Sweden. Trypsin (10150 BAEE U/mg solid), soybean trypsin inhibitor (SBTI, 10,000 BAEE U/mg protein), aprotinin (11.9 TlU/mg solid), and N-toluene sulfonyl-L-arginine methyl ester (TAME) were from Sigma Chemical Company, St. Louis, Missouri. H-D-Valyl-leucyl-arginine-p-nitroanilide 2HC1 (S-2266) was from Kabi Diagnostica, Stockholm, Sweden; 4,5-dihydroxy-2,7-naphthalenedisulfonic acid was from Wako Pure Chemical Industries, Osaka, Japan; L-Proryl-L-phenylalany-L-arginine-α-naphthylester (Pro-Phe-Arg-NE) was provided by Torii Pharmaceutical Ltd., Tokyo, Japan. Highly purified human urinary kallikrein (Lot No. HUK-80 MM) was kindly supplied by Prof. H. Moriya (Tokyo Science College).
A trace amount of albumin was found in this preparation. Urokinase, kininase, and caseinolytic activities were not detected. The specific activity of this human urinary kallikrein was 4,605 μmoles (S-2266 as substrate)/mg protein. Homogeneous porcine pancreatic kallikrein that was supplied by Ono Pharmaceutical Ltd., Osaka, Japan had a specific activity of 463 KU/mg protein. Pepstatin A was purchased from Protein Research Foundation, Minoh, Osaka, Japan.

**Preparation of Inactive Renin**

The pooled EDTA-treated plasma (400 ml) from normal subjects was dialyzed overnight against 25 mmol/liter Tris-HCl buffer (pH 7.4) containing 20 mmol/liter NaCl. The dialyzed sample was applied to a column of DEAE-Sepharose (5 x 35 cm) equilibrated with the above dialyzing buffer. After washing the column with the starting buffer, a linear gradient elution was carried out increasing the molarity of NaCl (20-220 mmol/liter). Inactive renin was eluted between 60 and 100 mmol/liter NaCl, as shown in figure 1. Two peaks of active renin were eluted between 105 and 125 mmol/liter NaCl and between 130 and 145 mmol/liter NaCl. The main peak was collected and used for further purification of active renin.

The fractions (No. 140-183) of inactive renin were pooled and concentrated with 70% ammonium sulfate. The precipitate was dissolved in 20 ml of distilled water and dialyzed against 50 mmol/liter Tris-HCl buffer (pH 7.0); 5 liters of the buffer was changed two times in 24 hours. The dialyzed sample was applied to a column of Blue-Sepharose CL-6B (2.6 x 30 cm), which had been equilibrated with the same buffer. After washing the column with the starting buffer, we carried out stepwise elutions with KCl in the starting buffer. The contaminating active renin was eluted with the starting buffer. Inactive renin was eluted with 0.1 mmol/liter KCl, as shown in figure 2. The fractions were dialyzed against 1 mmol/liter phosphate buffer (pH 7.0) overnight and then lyophilized. The purification steps and results obtained are summarized in table 1. This preparation of inactive renin showed no renin activity when incubated with sheep angiotensinogen for 4 hours in 0.1 mmol/liter phosphate buffer (pH 7.3) at 37° C, as described later.

**Preparation of Active Renin**

The main peak of DEAE-Sepharose column chromatography described above was further purified by concanavalin A-Sephalose column chromatography according to the method described previously. The specific activity increased 60-fold and was 0.8 ng AI/mg protein/hr. The final recovery was 48.5%. This preparation was used as active renin.

**Assay of Renin Activity**

Renin activity was measured by incubating the sample with 300 μl of sheep plasma in a total of 2 ml assay mixture containing 0.1 mol/liter phosphate buffer (pH 7.3), 10 mmol/liter EDTA and 3.4 mmol/liter 8-hydroxyquinoline at 37° C for 1 hour. The plasma was obtained from a sheep 3 days after bilateral nephrectomy. The reaction was stopped by placing the tube in a boiling water bath for 5 minutes. Angiotensin I generated was quantified by radioimmunoassay.

**Assay of Esterase Activity**

Esterolytic activity was measured with TAME as substrate by the modified method of Siegelman et al., with Pro-Phe-Arg-NE as substrate by the method of Hitomi et al., or with S-2266 as substrate.

**Activation of Inactive Renin**

The total volume of 500 μl incubation mixture contained the sample of inactive renin (100 μg protein),...
TABLE 1. Isolation of the Inactive Renin from Normal Human Plasma (400 ml)

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity activated by trypsin (ng AGI/hr)</th>
<th>Specific activity (ng AGI/mg/hr)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole plasma</td>
<td>27200</td>
<td>3540</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>DEAE-Sepharose column chromatography</td>
<td>1650</td>
<td>2780</td>
<td>1.68</td>
<td>78.5</td>
</tr>
<tr>
<td>70% ammonium sulfate precipitation</td>
<td>1150</td>
<td>2340</td>
<td>2.03</td>
<td>66</td>
</tr>
<tr>
<td>Blue-Sepharose column chromatography</td>
<td>8.2</td>
<td>1280</td>
<td>156</td>
<td>36</td>
</tr>
</tbody>
</table>

trypsin (2 \( \mu \)g), 0.1 mole/liter phosphate buffer (pH 7.3) and 3 mmol/liter CaCl2. The reaction was carried out at 37° C for 10 minutes. The reaction was stopped by adding SBTI in a concentration five times greater than trypsin. This quantity of SBTI completely inhibited trypsin activity as judged by complete inhibition of esterolytic activity (TAME as substrate). Then the reaction mixture was transferred to the renin-angiotensinogen reaction system described above. Parallel incubation of the same amounts of trypsin, SBTI, and sheep plasma at 37° C for 1 hour served as blank. No renin activity was detected in this control experiment.

Treatment of Inactive Renin with Glandular Kallikrein

The total volume of 500 \( \mu \)l incubation mixture contained the sample of inactive renin (100 \( \mu \)g protein), human urinary kallikrein (3, 6, 12, 30 and 100 \( \mu \)g) or pig pancreatic kallikrein (4, 8, 16, 40, and 100 \( \mu \)g), 0.1 mole/liter phosphate buffer (pH 7.3). The reaction was carried out at 37° C for 30 minutes. Then the reaction was stopped by adding aprotinin in a concentration five times greater than the kallikrein used. This quantity of aprotinin completely inhibited kallikrein activity, as judged by complete inhibition of esterolytic activity (Pro-Phe-Arg-NE and S-2266 each as substrate). After complete inhibition of kallikrein activity, renin activity in the reaction mixture was measured using sheep plasma as described above. Control reactions containing the same amounts of kallikrein, aprotinin, and sheep plasma were also carried out. No renin activity was detected.

Treatment of the Trypsin-Activated Renin with Glandular Kallikrein

The trypsin-activated renin (17.6 ng Al/hr: 0.1 mg protein) that was separated from trypsin and SBTI by gel filtration on Ultrogel AcA 44 was incubated with human urinary kallikrein (0.03–0.3 units: 6–60 \( \mu \)g) or pig pancreatic kallikrein (2.7–27 KU: 8–80 \( \mu \)g) in 1 ml of 0.1 mole/liter phosphate buffer (pH 7.3) at 37° C for 30 minutes. The reaction was stopped by adding aprotinin in a concentration five times greater than the kallikrein used. Then the reaction mixture was transferred to the renin-angiotensinogen reaction system described above.

Gel Filtration

Samples of 3 ml containing three marker proteins, bovine serum albumin, ovalbumin, and chymotrypsinogen, were applied to a 2.5 x 90 cm column of Ultrogel AcA 44 equilibrated with 0.1 mole/liter phosphate buffer (pH 7.0) containing 0.1% NaN3 at 4° C. Fractions of 3.25 ml were collected with an upward flow rate of 20 ml/hr. The column was calibrated using blue dextran (Mr 2,000,000), aldolase (Mr 158,000), bovine serum albumin (Mr 67,000), ovalbumin (Mr 45,000), chymotrypsinogen (Mr 25,000) and SBTI (Mr 20,000).
Isoelectric Focusing

Isoelectric focusing was carried out using an LKB 110 column by the method described previously.\textsuperscript{21}

Determination of Km and Ki Values

Km values of trypsin-activated renin, trypsin-activated kallikrein-treated renin, and active renin in plasma were determined using the Lineweaver-Burk plot with six different concentrations of sheep angiotensinogen (final concentrations between 0.122 and 0.035 \(\mu\)mole/liter) in 0.1 mole/liter phosphate buffer at pH 7.3 (total volume, 1 ml).

Ki values for pepstatin A of trypsin-activated renin, trypsin-activated kallikrein-treated renin, and active renin were determined for two substrate concentrations (0.168 \(\mu\)mole/liter and 0.056 \(\mu\)mole/liter) in 0.1 mole/liter phosphate buffer (total volume, 1 ml) by the Dixon plot.\textsuperscript{23} Because of the low solubility of pepstatin A at neutral pH, the reaction was carried out at pH 6.0. Pepstatin A, to final concentrations between 0.5 and 3.0 \(\mu\)moles/liter, was added to the reaction mixtures.

Protein Estimation

Protein was measured by the method of Lowry et al.,\textsuperscript{26} with bovine serum albumin as the standard.

Results

Isolated Inactive Renin and Its Activation

The specific activity of the isolated inactive renin was increased approximately 1200-fold as compared with the starting plasma and was 156 ng Al/mg protein/hr when activated by trypsin (table 2). Neither esterase activity (TAME, Pro-Phe-Arg-NE, and S-2266, each as substrate) nor kallikrein-inhibiting activity (S-2266 as substrate) could be detected in this preparation (200 \(\mu\)g). The activity of inactive renin (100 \(\mu\)g/ml) in 0.1 mole/liter phosphate buffer (pH 7.0) containing 0.1% NaN3 was preserved within a week at 4\(^\circ\)C, when it was activated by trypsin. It was not activated spontaneously by cold storage at 4\(^\circ\)C within a week, whereas the inactive renin fraction after DEAE-Sepharose column chromatography was often activated spontaneously by cold storage at 4\(^\circ\)C within a week. Treatments of the inactive renin preparation with dithiothreitol (50 mmoles/liter), NaCl (3 moles/liter) and sodium dodecyl sulfate (0.005\%\) did not cause significant activation.

This inactive renin was activated by trypsin, but not by glandular kallikrein from human urine and pig pancreas. The activity of trypsin-activated renin (156 ng Al/hr) was not influenced when it was further treated with glandular kallikrein (human urinary kallikrein: 30–300 \(\mu\)g; pig pancreatic kallikrein: 40–400 \(\mu\)g). More than 500 \(\mu\)g of human urinary or pig pancreatic kallikrein reduced the activity of trypsin-activated renin with dependence of dose. These results are shown in table 2.

Change of Apparent Molecular Weight of Inactive Renin

An apparent molecular weight of the inactive renin was determined to be 54,000 ± 1900 (SD, \(n = 4\)) daltons by gel filtration. The inactive renin was activated, and the apparent molecular weight decreased to 48,000 ± 1500 daltons (\(n = 5\)). When the trypsin-activated renin was treated with human urinary or pig pancreatic kallikrein, the apparent molecular weight fell to 43,000 ± 1500 (\(n = 5\)) daltons, which was identical with that of naturally occurring active renin in plasma (43,000 ± 800, \(n = 4\)) (fig. 3).

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**TABLE 2. Activation of the Inactive Renin by Trypsin and Kallikrein**

<table>
<thead>
<tr>
<th>Renin</th>
<th>Renin activity (ng AGI/mg/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactive renin (1 mg)</td>
<td>ND</td>
</tr>
<tr>
<td>Trypsin (20 (\mu)g)</td>
<td>156</td>
</tr>
<tr>
<td>Human urinary kallikrein (30–1000 (\mu)g)</td>
<td>ND</td>
</tr>
<tr>
<td>Hog pancreatic kallikrein (40–1000 (\mu)g)</td>
<td>ND</td>
</tr>
<tr>
<td>Trypsin-activated renin (1 mg)</td>
<td>156</td>
</tr>
<tr>
<td>Human urinary kallikrein (120 (\mu)g)</td>
<td>158</td>
</tr>
<tr>
<td>Hog pancreatic kallikrein (160 (\mu)g)</td>
<td>152</td>
</tr>
</tbody>
</table>

ND = not detected.

The activation reaction by trypsin was carried out for 10 minutes at 37\(^\circ\)C. In the case of kallikrein treatment, the reaction was carried out for 30 minutes at 37\(^\circ\)C.

Each value is the mean of three or five determinations.

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**FIGURE 3.** Apparent molecular weight determination of inactive renin, trypsin-activated renin, trypsin-activated kallikrein-treated renin, and active renin in plasma by gel filtration on Ultrogel AcA 44 column. Kav was plotted against the logarithm of molecular weight. • inactive renin; □ trypsin-activated renin; △ trypsin-activated kallikrein-treated renin; ○ active renin in plasma. BSA = bovine serum albumin; OA = ovalbumin; ChTN = chymotrypsinogen; SBTI = soybean trypsin inhibitor.
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FIGURE 4. Lineweaver-Burk plots of trypsin-activated renin, trypsin-activated kallikrein-treated renin, and active renin in plasma. The reaction was carried out in 0.1 mole/liter phosphate buffer (pH 7.3) with sheep angiotensinogen (final concentrations between 0.122 and 0.035 μmole/liter) at 37° C for 1 hour. (●) active renin in plasma; (○) trypsin-activated kallikrein-treated renin; (▲) trypsin-activated renin.

Km Value

Km values of active renin and renin-activated kallikrein-treated renin were identical: 60 ± 2 (SD, n = 3) nmoles/liter, at pH 7.3. However, trypsin-activated renin had a slightly but significantly higher Km value of 89 ± 3 (n = 3) nmoles/liter than that of the active renin (fig. 4).

Ki Value for Pepstatin A

The inhibitor constants (Ki) of pepstatin A for active renin and trypsin-activated kallikrein-treated renin were identical, 2.6 ± 0.06 (SD, n = 3) and 2.6 ± 0.11 (n = 3) μmoles/liter at pH 6.0, respectively. On the other hand, the Ki value for trypsin-activated renin was 5.0 ± 0.2 (n = 3) μmoles/liter. The type of inhibition was competitive in all cases.

pH Profile

The pH profiles for angiotensin formation of active renin, trypsin-activated renin, and trypsin-activated kallikrein-treated renin are shown in fig. 5. The active renin and trypsin-activated kallikrein-treated renin showed very similar profiles, but the profile was different for trypsin-activated renin.

Isoelectric Point

The inactive renin was subjected to the preparative isoelectric focusing. After the electrofocusing, each fraction was treated with suitable concentrations of trypsin. The main activity peak appeared at pH 5.14 at 4° C. Two additional small peaks were observed at pH 4.90 and pH 5.25. When the trypsin-activated renin was subjected to electrofocusing, only one pI value of 5.06 was obtained. This value shifted to a more acidic one (pH 4.82) on further treatment with human urinary kallikrein. The main peak of active renin appeared at pH 5.20 with a shoulder at pH 5.02. Profiles of the isoelectric focusing are shown in figure 6.

Discussion

There is ample evidence that human plasma contains inactive renin. Yokosawa et al.16 and Atlas et al.27 have separated this inactive renin from active renin. In the present paper, we also separated the inactive form of renin from active renin by two steps, DEAE-Sepharose and Blue-Sepharose column chromatographies. This preparation was a completely inactive form and had neither kallikrein activity nor kallikrein inhibiting activity.

An apparent molecular weight of the inactive renin was estimated to be 54,000 daltons by gel filtration on Ultrogel AcA 44. This value is very similar to those of inactive renin in normal or pregnancy plasma.16, 28-31 Day et al.,32 Hsueh et al.,33 and Day and Morris34 have reported high molecular weight forms of inactive renin more than 60,000 daltons in normal plasma and plasma from patients with renal carcinoma or diabetes mellitus. Almost all reports indicate that the molecular weight of plasma inactive renin does not change after treatment with acid or proteolytic enzymes. Recently, Atlas et al.31 have reported that the apparent molecular weight of trypsin-activated renin was smaller than that of the inactive renin.

In our experiment, also, the apparent molecular weight of the inactive renin decreased to 48,000 daltons after treatment with trypsin. Furthermore, when the trypsin-activated renin was treated with glandular kallikrein, the molecular weight fell to 43,000 daltons, which was the same molecular weight as a native form of active renin purified partially from normal human plasma. When the inactive renin was activated by trypsin or the trypsin-activated renin was treated with glandular kallikrein, the apparent molecular weights of the inactive renin and the trypsin-activated renin de-
increased, resulting in the changes of their net charges. This was confirmed by isoelectric focusing of the inactive renin, the trypsin-activated renin and the trypsin-activated kallikrein-treated renin. The trypsin-activated renin differed from a native form of active renin and the trypsin-activated kallikrein-treated renin with respect to molecular weight, pi value, Ki value for pepstatin A, and pH profile for angiotensin generation. Every parameter of the trypsin-activated kallikrein-treated renin, except pi value, coincided with that of naturally occurring active renin in plasma.

It has been reported that pH optimum and Km value of acid-treated, cryoactivated, or trypsin-activated renin appears to be similar to that of naturally occurring active renin in plasma.3, 33, 35 These experiments were carried out using plasma that endogenously contained various proteolytic enzymes and their inhibitors. Even if these proteolytic enzymes in plasma were inactive, they might be activated after acid, cryo, or trypsin treatment. Thus, activated proteolytic enzymes in plasma might cleave small peptides from the activated renin that makes it similar to a native form of active renin in plasma. Since the activation reaction of inactive renin employed with unfractionated plasma is complicated, the data obtained often result in confusing interpretations.

Our results indicate that the inactive renin is of a high molecular weight form (54,000) and a large part of the active renin in plasma is of small form (43,000). In this experiment we could not completely eliminate the possibility that the inactive renin preparation was a renin-inhibitor complex. But treatments of the inactive renin by procedures that cause dissociation of the renin-inhibitor complex did not activate the inactive renin. Thus, it seems that the trypsin-activated renin is an intermediate form of a degradative pathway of inactive renin in plasma. Glandular kallikrein converts this trypsin-activated renin (48,000) to low molecular weight active renin that is very similar to naturally occurring active renin in plasma. Although further investigation is required before the trypsin-activatable inactive renin in human plasma can be classified as a renin precursor, our results support the idea that the inactive renin in human plasma is a prorenin.

Acknowledgment
We thank Professor H. Moriya (Tokyo Science College) for his gift of human urinary kallikrein.

Figure 6. Isoelectric focusing profiles. Samples (3–5 ml) of 1% Ampholine at pH 4–6 (containing 0.1% Ampholine, pH 6–8 to obtain a more even distribution of conductivity between the electrodes) were applied to an LKB 110 column. Renin activity (●) and pH (X) in 2.6 ml fractions after 48 hours of focusing were measured. A. Inactive renin activated by trypsin (2 μg/ml) after focusing. B. Trypsin-activated renin. C. Trypsin-activated kallikrein-treated renin. D. Active renin in plasma.
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