The Storage Form of Human Renal Renin

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SUMMARY We isolated renin granules from cadaver kidneys using discontinuous sucrose density gradient centrifugation, and investigated the storage form of the renin from these granules. Approximately 23% of the total renin activity in the original homogenate was obtained from the surface phase between 1.6 and 1.7 M sucrose (Fraction 6). Granule renin extracted from the granules in Fraction 6 was separated into active and inactive renin using pepstatin affinity chromatography. Only the active renin had an affinity for pepstatin. The inactive renin, albeit activated by trypsin, was little activated by acidification. The proportion of inactive renin was about 25% of the total granule renin (active renin + inactive renin). Trypsin concentrations over 10 μg/ml resulted in a decrease in the renin activity of the trypsin-activated renin, but the enzymatic activity of active renin was decreased by trypsin. With gel filtration, the inactive renin revealed a single peak, and the molecular weight (MW) was 48,000. The active renin had a MW of 44,000. The inactive renin could be activated by trypsin without an apparent change in molecular weight. (Hypertension 4: 211-218, 1982)

KEY WORDS • acidification • active renin • inactive renin • renin granules • trypsin

I

In current concepts of the biochemistry of renin, the idea of multiple forms of renin has gained a wide acceptance. There is a high molecular weight form of over 50,000 and an inactive form that can be activated by acidification or with a proteolytic enzyme. These have been the subject of several reviews.1-8

Renin is stored in specific granules in the juxtaglomerular cells.4 Data concerning the storage of renin in dogs,6-7 rats,8-9 and pigs10 have been reported. Funakawa et al.,8 and our own group6,7 found that the storage form of dog renin has a low molecular weight of 40,000 and that this form of renin could not be activated by acidification. Sagnella et al. reported similar findings in the case of rats8 and pigs.10

We have now isolated renin granules from the human kidney. We report our findings and discuss the storage form of renin herein.

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Material and Methods

Kidney Tissue Homogenization

Kidneys from 10 human cadavers were obtained at autopsy within 4 hours of death. The patients ranged in age from 55 to 83 years; all had died of causes not related to kidney disease and had no past history of kidney-related disorders. Macroscopic and microscopic examinations of the kidneys revealed no pathological findings. With a razor blade, 20 g of renal cortex was cut into fine, small slices, which were put into 80 ml of ice-cold 0.45M sucrose; the preparation was then homogenized in a Potter-Elvehjem homogenizer with a loose-fitting pestle, at 800 rpm for 40 seconds at 4°C.

After centrifuging the homogenate at 500 g for 10 minutes, we used the supernatant (original homogenate) for fractionation by discontinuous sucrose density gradient centrifugation. The gradient was prepared by layering 7 ml each of sucrose solution with six different concentrations (1.2 to 1.7 M with 0.1 M intervals) in three respective centrifuge tubes. Then 10 ml of the original homogenate was layered on top of 42 ml of the sucrose density gradient, and the preparations were centrifuged at 60,000 g for 90 minutes using a RPS 25-2 rotar, Hitachi model S5P-72, ultracentrifuged at 4°C. After centrifugation, the
The density gradient was fractionated according to the principle described by Hogeboom and Kuff.11 The gradient was displaced upward by pumping 1.8 M sucrose into the bottom of the tube at a flow rate of 1.5 ml/min. The eluate was monitored at 280 nm, and samples were obtained at 42-second intervals using an ISCO model 1200 fraction collector. Seven fractions were recovered; Fraction 0 consisted of Samples 1 to 8 (volume = 8.4 ± 0.1 ml); Fraction 1, of Samples 9 to 13 (volume = 5.2 ± 0.1 ml); Fraction 2, of Samples 14 to 19 (volume = 6.3 ± 0.1 ml); Fraction 3, of Samples 20 to 26 (volume = 7.3 ± 0.1 ml); Fraction 4, of Samples 27 to 33 (volume = 7.3 ± 0.1 ml); Fraction 5, of Samples 34 to 40 (volume = 7.3 ± 0.1 ml); and Fraction 6, of Samples 41 to 50 (volume = 10.5 ± 0.1 ml). The sediment was resuspended in 7 ml of 0.45 M sucrose and used as Fraction 7. The supernatant and each fraction were analyzed for enzymes and protein.

Enzyme Activities
At 37°C for 30 minutes, we incubated tubes containing 0.1 ml portions of the diluted sample, 0.1 ml of sheep renin substrate prepared by the method of Skinner12 (maximum amount of angiotensin I (AI) that could be liberated per 100 pmole), and 0.8 ml of phosphate buffer (0.1M, pH 7.0), together with EDTA (10 mM), dimercaprol (3.2 mM), and 8-hydroxyquinoline sulfate (1.6 mM). At the end of 30 minutes, the reaction was stopped by immediate cooling. The remaining proteins bound to the column were eluted according to the method of Andrews.1' As a control, the samples were dialyzed separately against 0.05 M phosphate buffer (pH 7.0) for 4 hours.

Acidification
Acidification was carried out by a modification of the method of Skinner et al.6' Aliquots of the sample were dialyzed in Visking cellophane tubing (8/32) against 500 volumes of 0.05 M glycine/HCl buffer containing 0.1 M NaCl (pH 3.3) for 24 hours at 4°C and subsequently against 0.05 M phosphate buffer containing 0.1 M NaCl (pH 7.0) for 24 hours at 4°C. As a control, the samples were dialyzed separately against 0.05 M phosphate buffer (pH 7.0) for 48 hours.

Affinity Chromatography
Pepstatin-aminohexyl-Sepharose was prepared by the method of Murakami and Inagami;11 1 ml of the wet gel contained 1.2 μmole of covalently bound pepstatin. A column (1.6 × 30 cm) was equilibrated with buffer I. A mixture of granule renin was obtained from an equal volume of 10 experiments; 5 ml of the mixture was applied to the column, and the column was then thoroughly washed with buffer I. The remaining proteins bound to the column were eluted with 0.5 M Tris-acetate buffer (pH 7.5). Fractions of 5 ml were collected at a flow rate of 0.4 ml/min; 0.1 ml of each fraction was assayed for renin activity. Eluates from Fraction 4 to 13 (peak I), and eluates from Fraction 19 to Fraction 40 (peak II) were mixed, and peak II was further dialyzed against buffer I for 24 hours at 4°C. Both fractions were kept frozen at −70°C before use.

Trypsin Treatment
Trypsin (crystallized Type III, specific activity 10,200 BAEE units/mg protein, Sigma) was dissolved in buffer I, and 70 μl was added to 1330 μl of sample to acquire three trypsin concentrations, 1, 10, or 100 μg/ml respectively. After the sample had been preincubated for 5 minutes at 0°C or 25°C, respectively, incubation was carried out for six periods, 1, 5, 15, 30, 60 and 180 minutes. Then 200 μl of the sample were withdrawn at each period and transferred to the tube containing 10 μl of ice-cold soybean trypsin inhibitor (SBTI) (Type I-S, Sigma) at a concentration of 4 mg/ml. This quantity of SBTI was more than twice that required to inhibit the trypsin at a concentration of 100 μg/ml. For zero incubation time, after 190 μl of the sample has been preincubated for 5 minutes at
25°C or 0°C, a mixture of 10 μl for each trypsin concentration and 10 μl of the SBTI was added. These samples were diluted and assayed for renin activity.

A false renin concentration may be obtained due to the effects of trypsin on the assay system. The trypsin-SBTI mixture may produce Al from sheep renin substrate, and the trypsin may generate a substance that cross-reacts with Al antibody (immunoreactive material) from the sample. Trypsin-induced proteolytic enzymes in the sample may destroy the iodinated Al or the Al produced. To elucidate these factors, 0.1 ml of buffer I containing 10 μg of trypsin and 20 μg of SBTI was incubated for up to 90 minutes with 0.8 ml of buffer containing the inhibitor mixture and 0.1 ml of renin substrate, as described in Methods. The Al was not detected under this condition. Then, before incubation with the renin substrate, each trypsin-treated sample was incubated for up to 90 minutes with buffer containing the inhibitor mixture, without the renin substrate. Renin activity was not detected under this condition, in any sample. These results clearly show that there was no effect of trypsin on the assay system we used.

To the nontrypsin-treated sample, we then added 70 μl of buffer I to 1330 μl of the sample, and the procedures described above were carried out. The sample was assayed before incubation with the renin substrate. Al was not detected under this condition, in any sample.

Preparation of “Granule Renin”

Lysis of renin granules was performed by the method of Morimoto et al. and Funakawa and Yamamoto. Fraction 6 obtained by sucrose density gradient centrifugation was diluted with 1 vol of buffer I containing 0.2% Triton X-100, and the preparation was centrifuged at 100,000 g for 30 minutes. The supernatant was used as “granule renin” and frozen at −70°C before use.

Results

Fractionation by Density Gradient Centrifugation

The isolation of renin granules was investigated by comparing the centrifugal distribution to those of the other subcellular organelles of the renal cortex, as presented by their enzyme markers. Table 1 shows the average distribution of marker enzyme and protein distribution in each fraction obtained in 10 experiments. Acid phosphatase and glucose-6-phosphatase showed the highest activity in Fraction 1, which contained about 33% in the total recovered from all fractions, with about a 2.5-fold increase in specific activity over the original homogenate. Acid phosphatase showed another peak in Fraction 6, which had a lower peak than Fraction 1. Succinate dehydrogenase showed about 12% to 22% of the total recovered in each Fraction from 1 to 6, with about a twofold increase in specific activity over the original homogenate. On the other hand, renin activity showed the highest activity in Fraction 0, which contained 43.6% of the total recovered. Of Fractions 1 to 7, Fraction 6 had the highest renin activity, that is, about 23% of the total recovered. The specific activity of renin in Fraction 6 was 3.3 times that in the original homogenate, and the specific activity of other respective enzymes in this fraction was 1.6 to 2.2 times that in the original homogenate.

To assess the storage of renin in the granules, three tubes (A, B, and C) containing 1 ml of Fraction 6 were prepared. One ml of buffer I was added to Tubes A and B. Tube B was further frozen and thawed five times. To Tube C, 1 ml of buffer I containing 0.2% of Triton X-100 was added. These three tubes were then centrifuged at 100,000 g for 60 minutes, and the renin activity in the supernatant was measured. Table 2 shows a marked increase of renin activity by treatment of freezing and thawing or by treatment with Triton X-100.

Effect of Trypsin Treatment of Peaks I and II Separated by Pepstatin Affinity Chromatography

The granule renin was separated into two peaks by affinity chromatography (fig. 1). Peak I, eluted without adhering to the column and showed no renin activity. Peak II was eluted with 0.5 M Tris-acetate buffer pH 7.5. This fraction accounted for about 64% of the total renin activity of the granule renin applied to the column.

![Figure 1](http://hyper.ahajournals.org/DownloadedFrom)
Table 1. Enzyme and Protein Distribution in Fractions After a Sucrose Density Gradient Centrifugation

<table>
<thead>
<tr>
<th>Enzyme and Protein</th>
<th>Original homogenate</th>
<th>Fractions isolated by a sucrose density gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F0</td>
<td>F1</td>
</tr>
<tr>
<td>Renin a)</td>
<td>2.16 ± 0.40</td>
<td>1.67 ± 0.35</td>
</tr>
<tr>
<td>Acid phosphatase c)</td>
<td>3.03 ± 0.18</td>
<td>43.6 ± 1.6</td>
</tr>
<tr>
<td>Glucose-6-phosphatase a)</td>
<td>13.4 ± 0.69</td>
<td>130 ± 10.0</td>
</tr>
<tr>
<td>Succinate dehydrogenase c)</td>
<td>7.68 ± 0.59</td>
<td>1.87 ± 0.09</td>
</tr>
<tr>
<td>Protein b)</td>
<td>0.80 ± 0.03</td>
<td>43.6 ± 1.6</td>
</tr>
<tr>
<td>b)</td>
<td>12.43 ± 0.69</td>
<td>45.9 ± 0.7</td>
</tr>
<tr>
<td>c)</td>
<td>19.57 ± 1.14</td>
<td>101 ± 1.0</td>
</tr>
</tbody>
</table>

a) Specific activity of marker enzymes in the original homogenate and the isolated fractions. Units are n mole of angiotensin I/hr/mg protein for renin; µg of released inorganic phosphate from substrate/hr/mg protein for acid phosphatase and glucose-6-phosphatase; and decrease of absorbancy at 400 nm/hr/mg protein for succinate dehydrogenase.

b) Protein concentration (mg/ml) in the original homogenate and the fractions isolated.

c) Distribution of marker enzyme activities (fraction volume × enzyme activity) and protein contents (fraction volume × protein).

Figure 2 shows the renin activity of peak I with three trypsin concentrations for seven periods at 0°C and 25°C. With incubation up to 180 minutes, renin activity was not detected in nontrypsin-treated samples. Figure 2 a shows a marked increase of renin activity, as induced by trypsin. The maximum amount of trypsin-activated renin was obtained at a concentration of 10 µg/ml with incubation for 15 minutes at 0°C. The value was 125 pmoles of Al/ml/hr. A longer incubation led to a decrease in renin activity. Incubation at a concentration at 100 or 1 µg/ml of trypsin also led to an increase of renin activity and subsequent decrease of the enzyme activity. Loss of renin activity was more rapid with higher concentrations of trypsin. Incubation for 1 minute at 25°C produced the maximum activity, at each concentration of trypsin, and subsequent incubation produced a more rapid decrease of the enzyme activity than was seen at 0°C.

Table 2. Effect of Freezing and Thawing and Triton X-100 on Renin Activity of Fraction 6

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (not treated)</td>
<td>0.82</td>
<td>0.71</td>
<td>0.93</td>
<td>0.42</td>
<td>0.72 ± 0.15</td>
</tr>
<tr>
<td>B (freezing and thawing)</td>
<td>7.06</td>
<td>3.62</td>
<td>5.21</td>
<td>2.88</td>
<td>4.69 ± 0.93</td>
</tr>
<tr>
<td>C (Triton X-100)</td>
<td>7.91</td>
<td>4.01</td>
<td>5.98</td>
<td>3.03</td>
<td>5.23 ± 1.08</td>
</tr>
</tbody>
</table>

Table 3. Effect of Acidification on the Activity of Active and Inactive Renin

<table>
<thead>
<tr>
<th>Material</th>
<th>Treatment</th>
<th>pmole of Al/ml/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak I</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>(inactive renin)</td>
<td>pH 7.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pH 3.3</td>
<td>8.9 ± 1.0</td>
</tr>
<tr>
<td>Peak II</td>
<td>None</td>
<td>115 ± 4.5</td>
</tr>
<tr>
<td>(active renin)</td>
<td>pH 7.0</td>
<td>111 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>pH 3.3</td>
<td>113 ± 3.6</td>
</tr>
</tbody>
</table>

Values are means ± SE of triplicate assays. Peaks I and II were obtained following acidification, as described in Methods.
The inactive renin activated by trypsin at a concentration of 10 μg/ml and incubation for 15 minutes at 0°C was proportional to the time of incubation with sheep renin substrate, up to 90 minutes (fig. 3).

Figure 4 shows the effect of trypsin on the renin activity in peak II. Change in the renin activity in non-trypsin-treated samples was nil with incubation periods up to 180 minutes. The renin activity decreased, time dependently, at any temperature and at any concentration of trypsin. Decrease in the activity was more extensive at 25°C than at 0°C.

These results indicate that peak I contained inactive renin that was activated by trypsin and that the enzymatic activity of the subsequently activated renin was simultaneously decreased by trypsin. Peak II contained active renin only.

Effect of Acidification of Peak I (Inactive Renin) and Peak II (Active Renin)

Peaks I and II were separately dialyzed, as described in Methods. When peak I was dialyzed against neutral buffer for 48 hours, there was no evidence of renin activity. However, the acidified sample did have a renin activity of 8.9 pmole of Al/ml/hr, which indicates that a small amount of inactive renin was activated. On the other hand, when peak II was dialyzed against a neutral or acid buffer, the renin activity was little changed (table 3).

![Figure 3](http://hyper.ahajournals.org/)

**Figure 3.** Production of angiotensin I from sheep renin substrate by trypsin-treated peak I (○) and peak II (●).

Peak I was obtained following treatment with trypsin at a concentration of 10 μg/ml for 15 minutes at 0°C, as described in Material and Methods. The trypsin-treated preparation was incubated with renin substrate up to 90 minutes. The generated angiotensin I was subjected to radioimmunoassay. Peak II was not treated with trypsin.

![Figure 4](http://hyper.ahajournals.org/)

**Figure 4.** Effects of trypsin concentration, incubation temperature, and time on active renin in peak II. Procedures were the same as in figure 2 except that peak II was used instead of peak I. The angiotensin I was not detected in controls prior to incubation with renin substrate.
Molecular Weight of Inactive and Active Renin

When 1 ml of peak II was applied to a Sephadex G-75, the renin showed one peak that was eluted slower than ovalbumin and corresponded to MW 44,000 (fig. 5a).

Peak 1 was applied to gel chromatography, and the eluate was treated with trypsin. In preliminary experiments, 3 ml that eluted faster and 3 ml that eluted slower than ovalbumin were collected to determine the optimum condition of trypsin treatment. When both samples were treated with trypsin in concentrations of 1 or 10 µg/ml with incubations of 1, 5, 15, 30, or 60 minutes at 0°C, both were activated to a greater extent than with a concentration of 1 µg/ml for 15 minutes. Therefore, the eluate was treated by trypsin in a concentration of 1 µg/ml for 15 minutes at 0°C. The inactive renin eluted faster than ovalbumin, and the peak corresponded to MW 48,000 (fig. 5b). The recovery of inactive renin from the column was about 76% of the total renin activity obtained from a sample activated by trypsin at a concentration of 10 µg/ml and with incubation for 15 minutes at 0°C, before application to column. When the inactive renin was activated by trypsin at a concentration of 10 µg/ml and an incubation of 15 minutes at 0°C, and then applied to the column, the enzymatic activity was eluted faster than ovalbumin and corresponded to MW 48,000 (fig. 5c). Recovery of trypsin-activated renin from the column was 79% of the total renin activity applied to the column. In a preliminary examination, the mixture of trypsin and trypsin inhibitor exerted no appreciable effect on the gel filtration profile.

These results suggest that the molecular weight of inactive human renin is 48,000 and that of active renin, 44,000, and that the activation occurs without apparent change in molecular weight.

Discussion

This report is the first documentation of the separation of renin granules from human renal cortex and the storage form of renin. With discontinuous sucrose density centrifugation, renin granules were mainly recovered in the fraction corresponding to 1.7 M sucrose. This finding suggests that this renin is probably stored in the renin granules. Most of the acid phosphatase, glucose-6-phosphatase, and succinate dehydrogenase was equilibrated in the upper layers of the gradient above the renin granule-rich fraction.

The renin activity was mainly recovered in the supernatant fraction (Fraction 0). Renin granules are reportedly fragile and, therefore, disruption probably occurs during homogenization of the cortical slices. As this fractionation method disrupts a considerable number of renin granules, and the renin granule fraction is contaminated with other organelles, other procedures for preparation of renin granules should be designed.

Histochemical studies revealed certain similarities between renin granules and lysosomes. Recent studies on subcellular fractionations by isopycnic or zonal centrifugation revealed a distinct distribution between these renin granules and lysosomes. In the subcellular fractionation of human kidney cortex, lysosomes showed a main peak in the fraction corresponding to 1.2 M sucrose (Fraction 1) in contrast with findings of renin granules to 1.7 M sucrose fraction. However, the density seems to be low for lysosomes, and electron microscopic examination showed that this fraction did contain a considerable amount of vacuole formation and membrane fragments. This can probably be attributed to rupture of the intact lysosomes.

In our experiments, we used kidneys obtained no later than 4 hours after death, to minimize the autolytic destruction of renin granules. Care was taken to exclude tissue with obvious renal pathology. In preliminary experiments, when the original homogenate was obtained from the kidney cortex over 12 hours after death and was then separated into soluble and insoluble fractions by ultracentrifugation, almost all the renin activity was recovered from the soluble fraction. The renin granules appear to undergo rapid disruption with the autolysis following death.

![Figure 5](http://hyper.ahajournals.org/)

**Figure 5.** Gel filtration of peak II (a), peak 1 (b), and trypsin-treated peak 1 (c) on a Sephadex G-75. In (a), peak II was applied to a column and the eluate was assayed for renin activity. In (b), eluates after gel filtration were treated with (●) and without (○) trypsin at a concentration of 1 µg/ml for 15 minutes at 0°C, and then assayed for renin activity. In (c), peak 1 treated with trypsin at a concentration of 10 µg/ml for 15 minutes at 0°C was applied to the column and the eluates were assayed for renin activity.
The granules contained an active and inactive form of renin. These renins could be completely separated by affinity chromatography, according to a modification of the method of Yokosawa et al.18 The inactive renin was not due to an assay artifact. Trypsin did not produce AI from sheep renin substrate and did not generate immunoreactive material. The inactive renin was proportional to the time of incubation with renin substrate. The proportion of inactive renin was calculated to be about 25% of the total renin activity in the renin granules (inactive renin activated by trypsin in peak I + active renin before application to the affinity column).

The concentration of trypsin required for maximum activation of inactive renin in the granules was much lower than the concentrations in human plasma. Atlas et al.19 reported that a low concentration of trypsin-activated inactive renin in plasma from which endogenous protease inhibitors containing trypsin inhibitor had been removed. Sealy et al.20 found that, although the hydrolytic ability of trypsin was more potent at 25°C than at −4°C, the rate of activation of inactive renin was in contrast with the hydrolytic activity. These data indicate that a high concentration of trypsin is required to counteract the trypsin inhibitors in the plasma. In the present study, as this reaction was more rapid at 25°C than at 0°C, inactive renin seems to be activated by trypsin in proportion to the hydrolytic activity. The amount of trypsin inhibitors in the renin granules seems to be scanty, as compared with levels in the plasma. On the other hand, activity of the active renin decreased to a greater extent when incubations were carried out with trypsin at higher concentrations and at 25°C. Thus, the active renin is also probably destroyed by trypsin.

Acid activation of the inactive renin in the granules produced about 7% of the increase induced by trypsin. Hseuh et al.21 reported that inactive renin was completely activated either by acidification or by trypsin. In our study in dogs, inactive renin was not activated by acidification of the granules. Although the inactive renin in the renin granules was activated by trypsin (Kawamura et al., unpublished data). Trypsin appears to be a potent tool for activation of inactive renin in renin granules; however, care must be taken as both active renin and trypsin-activated renin may be destroyed by trypsin itself.

The MW of inactive renin, trypsin-activated renin, and active renin was estimated by gel chromatography. The inactive renin probably has a higher MW and is activated without apparent change in molecular weight. This result is similar to findings in the case of normal human plasma.22,23, although the MW of inactive renin in human plasma is over 50,000. Since inactive and active renin in the granules eluted in similar positions, however, determination of the differences in MW would require other procedures. The renin granules contained 25% of inactive renin, thereby suggesting that inactive renin in human plasma originates from the kidney. Further studies are underway to elucidate mechanisms of the activation of inactive renin in human renin granules.

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