The Storage Form of Renin in Renin Granules from Rat Kidney Cortex

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SUMMARY Renin granules were partially purified from rat kidney cortex, and a storage form of renin in the granules was examined. Renin granules were isolated by discontinuous Percoll density gradient centrifugation followed by continuous Percoll density gradient centrifugation. The partially purified fraction was free from mitochondria and microsomes, as judged by the absence of marker enzymes of these organelles, but contained some lysosomal enzyme activities. The specific renin activity was 0.58 mg angiotensin I/hr/mg protein, 500 times as active as the original homogenate. Immunochemical staining with specific antisera against rat kidney renin revealed that about 10% of the granules recovered in the partially purified fractions were stained strongly. The stored renin was not activated either by acidification or by trypsin treatment, indicating that stored renin was in the fully active form. By sodium dodecyl sulfate gel electrophoresis, the stored renin had two different molecular weights, 38,000 and 36,000, and these molecular weights were not reduced by dithiothreitol or 2-mercaptoethanol, suggesting that these renins are single-chain types as opposed to the two-chain type found in male mouse submaxillary gland. These results suggest that active renins with two different molecular weights may be released from renin granules of juxtaglomerular cells.

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KEY WORDS • renin granules • purification • rat renin • storage form • Percoll density gradient centrifugation

THE secretion of renin from juxtaglomerular cells is regulated by a variety of stimuli that presumably are transmitted by the baroreceptor, the neurogenic receptor, and the macula densa receptor. It is generally accepted that calcium acts as a negative modulator of renin secretion, and as it does in the release of parathyroid hormone. Although much evidence indicates that renin is stored in specific granules of juxtaglomerular cells, little is known about the renin secretory process because of the extremely low number of juxtaglomerular cells in the kidney. Renin constitutes only 0.01% of kidney proteins.6

Recently, the presence of more than one pathway of renin secretion has been suggested by several groups of investigators.5,6 In one pathway, renin is stored in granules before release, while in another pathway newly synthesized renin is secreted directly. To gain further insight into the mechanism of the renin secretory process, information must be obtained on the biochemical nature of the stored renin. In most studies, renin granules have been isolated by a discontinuous sucrose density gradient centrifugation method from human,9 dog,10 rabbit,11 and rat12 kidneys. The specific activity of renin granule preparation was enriched at most five to 20 times as compared with that of the homogenate of the whole kidney cortex. We have attempted to obtain a higher degree of purification by devising a different procedure. Thus, the goal of the present report was to isolate renin granules using this new method and to study the storage forms of renin.

Materials and Methods

Animals and Fractionation of Homogenate

Male Sprague-Dawley rats, weighing 180 to 220 g, were fed Purina rat chow (St. Louis, MO, USA) containing 0.39% sodium and allowed free access to tap water. Rats were anesthetized with sodium pentobarbital (5 mg/100 g body weight i.p.), and an abdominal incision was made to expose the kidneys. Kidneys were removed after perfusion with isotonic saline, and the kidney cortex was minced and gently homog-
enized with 0.45 M sucrose (1:7 wt/vol). The homogenate was centrifuged at 500 g for 10 minutes, and 4 ml of the supernatant (original homogenate) then was placed on a discontinuous Percoll density gradient solution (Sigma Chemical, St. Louis, MO, USA). The Percoll solution was made isosmotic with 0.25 M sucrose (final concentration). The gradient consisted of three 8-ml layers of 1.07, 1.11, and 1.15 g/ml Percoll solutions. After centrifugation at 40,000 g for 15 minutes, the gradient had three visible bands. The gradient was separated into four fractions, F0 (volume, 4.0 ml), F1 (4.0 ml), F2 (7.0 ml), and F3 (11 ml), from the top of the layers of the solution by using an ISCO Model 185 Fractionator (Instrumentation Specialties, Lincoln, NE, USA). Then, 10 ml of F3 solution was added to a polycarbonate tube (1.4 × 13 cm), centrifuged at 20,000 g for 90 minutes using a JA-20 rotor (Beckman, Palo Alto, CA, USA), and fractionated into 10 fractions (1 ml each) by pipetting. Renin granules were lysed by mixing the granule–containing solution with 0.1 volume of phosphate buffer (0.1 M, pH 7.0) containing 1% Triton X-100. The mixture was centrifuged at 100,000 g for 120 minutes, and the supernatant was used for renin activity assay. Density was calculated from refractive index using an Abbe refractometer (Fisher Scientific, Pittsburgh, PA, USA).

**Estimation of Molecular Weight**

Gel filtration was performed on a Sephadex G-75 column (Pharmacia, Piscataway, NJ, USA); 1 ml of a diluted sample containing molecular weight markers was applied to a Sephadex G-75 column (1.6 × 90 cm) that had been equilibrated with phosphate buffer (0.1 M, pH 7.2) containing 0.1 M NaCl, and 1 ml fractions were collected for measurement of renin activity.

The molecular weight of renin was estimated by sodium dodecyl sulfate gel electrophoresis followed by the Western blotting technique as detailed by Burnette. Samples were electrophoresed in 11% sodium dodecyl sulfate–polyacrylamide gel according to the method of Laemmli. The proteins were transferred electrophoretically to a nitrocellulose sheet (5 hours at 3 V/cm in 40 mM Na phosphate buffer, pH 6.5). The sheet was treated with anti-rat kidney renin antibody at a 1:500 dilution, washed thoroughly, then treated with radiiodinated protein A (70–100 µCi/µg; New England Nucleic, Boston, MA, USA) at a concentration of 5 × 10⁵ cpm/ml. After extensive washing, the sheet was exposed at −70°C to Kodak XAR5 film (Rochester, NY, USA). Before application to the electrophoresis gel, the sample was divided into three portions and pretreated as follows. The first sample was heated for 3 minutes at 90°C and then centrifuged at 13,000 g for 5 minutes. The supernatant then was collected for the electrophoresis. The second sample was heated to 90°C in the presence of 5% 2-mercaptoethanol and then was treated in the same way as the first sample. The third sample was incubated at 25°C for 1 hour in the presence of 10 mM dithiothreitol. The sample was then filtered through a Diafilter membrane YM 10 (Amicon, Danvers, MA, USA) with 50 volumes of tris(hydroxymethyl) aminomethane (Tris) HCl buffer (20 mM, pH 7.5) containing 1 mM dithiothreitol. The dithiothreitol-treated sample was then treated with mercaptoethanol in the same way as the second sample.

**Measurement of Enzyme Activities and Protein Content**

Renin activity was measured by the rate of formation of angiotensin I (ANG I) from rat angiotensinogen as described previously. The ANG I produced was assayed by radioimmunoassay according to the method of Haber et al. Succinate dehydrogenase, a marker enzyme of mitochondria, was assayed by the method of Slater and Bonner. Glucose-6-phosphatase, a marker for microsomes, and acid phosphatase, a lysosomal marker, were assayed according to the method of Morimoto et al. Acid protease, another lysosomal marker, was determined by a modification of the method of Williams and Lin. In preparatory experiments, we confirmed that these enzyme activities were not influenced by the addition of Percoll solutions under conditions used in the present assay system. The protein concentration was determined by the method of Lowry et al. using bovine serum albumin as the standard. As Percoll caused a background coloration with the reagent for the protein assay, each sample value was corrected for background obtained with a Percoll solution of an identical concentration not containing the kidney homogenate.

**Activation of Inactive Renin**

Activation of inactive renin was performed separately by acidification and by trypsin treatment. Acidification was performed as reported previously. Samples were diluted with 4 volumes of phosphate buffer (50 mM, pH 7.0) containing 0.1 M NaCl. Then, 0.5 ml of sample was dialyzed against glycine HCl (50 mM, pH 3.3) containing 0.1 M NaCl for 24 hours and subsequently against phosphate buffer (50 mM, pH 7.0) containing 0.1 M NaCl for 24 hours. As a control, the samples were dialyzed against the neutral buffer for 48 hours. Trypsin treatment was performed as reported previously. To remove active renin, 5 ml of sample was applied to a pepstatin Sepharose column. The pass-through fraction was concentrated to 5 ml, and the aliquot was incubated with trypsin (100 µg/ml) up to 60 minutes. After the reaction was stopped by the addition of soybean trypsin inhibitor, the renin activity of each sample was measured.

**Immunocytochemistry**

For immunocytochemistry analyses, 20 volumes of 0.45 M sucrose were added to a fractionated sample and the solution was centrifuged at 5,000 g for 10 minutes. The pellets were fixed for 3 hours at 25°C in phosphate buffer (0.1 M, pH 7.4) containing 4% paraformaldehyde, 0.25% glutaraldehyde, and 0.3% picric acid. The pellets were then dehydrated in dimethyl formamide and embedded in Lowicryl K 4M (Polysciences,
Table 1. Enzyme and Protein Distribution in Fractions After Discontinuous Percoll Density Gradient Centrifugation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Original homogenate</th>
<th>F0</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renin (µg ANG I/hr)</td>
<td>95.7 ± 5.63</td>
<td>33.5 ± 2.48</td>
<td>5.6 ± 0.42</td>
<td>7.6 ± 0.70</td>
<td>50.6 ± 4.73</td>
</tr>
<tr>
<td>Acid protease (10^3 cpm/hr)</td>
<td>584 ± 13.2</td>
<td>102 ± 8.50</td>
<td>151 ± 12.1</td>
<td>153 ± 11.9</td>
<td>32.8 ± 2.20</td>
</tr>
<tr>
<td>Acid phosphatase*</td>
<td>1580 ± 88.3</td>
<td>779 ± 53.9</td>
<td>424 ± 34.5</td>
<td>350 ± 32.7</td>
<td>33.4 ± 3.06</td>
</tr>
<tr>
<td>Glucose-6-phosphatase*</td>
<td>883 ± 21.6</td>
<td>246 ± 9.87</td>
<td>594 ± 37.1</td>
<td>9.59 ± 0.29</td>
<td>0</td>
</tr>
<tr>
<td>Succinate dehydrogenase†</td>
<td>266 ± 16.5</td>
<td>19.2 ± 2.60</td>
<td>52.4 ± 6.4</td>
<td>137 ± 13.9</td>
<td>0</td>
</tr>
<tr>
<td>Protein (mg)</td>
<td>81.7 ± 0.99</td>
<td>36.2 ± 1.40</td>
<td>31.1 ± 1.30</td>
<td>14.2 ± 0.30</td>
<td>2.20 ± 0.20</td>
</tr>
</tbody>
</table>

Values are means ± SE of five experiments. Enzyme activities and protein contents in 4 ml of original homogenate
in each fraction volume. F = fraction; ANG I = angiotensin I.

*Measured as micrograms of released inorganic phosphate from substrate per hour.
†Measured as absorbancy at 400 nm/hr.

Results

Renin granules were isolated by discontinuous and then continuous Percoll density gradient centrifugation. Table 1 and Figure 1 show the average distributions of marker enzymes and proteins in each fraction separated by the discontinuous centrifugation. Approximately 53% of the total renin activity was contained in F3 (interphase between 1.11 and 1.15 g/ml).

Glucose-6-phosphatase and succinate dehydrogenase activities were not detected, while 5% of the total lysosomal marker enzyme activity was in this fraction. The specific activity of renin in F3 was 23.0 µg ANG I/hr/mg protein, which was 20 times that of the original homogenate. Figure 2 shows enzyme activities and protein concentration of fractionated samples after continuous Percoll density gradient centrifugation of 10 ml of F3. Renin activity was found to peak in Fraction 8 (partially purified renin granule fraction), which corresponded to a density of 1.138 g/ml. The specific activity of renin in this fraction was 0.58 ± 0.08 mg ANG I/hr/mg protein, which was 500 times that of the original homogenate. Of the total renin activity in F3 applied to the continuous gradient, 24% was present in Fraction 8. Thus, 13% of the total renin activity in the original homogenate was recovered in this peak fraction. This figure contrasts with an overall recovery of 0.4% of acid protease activity in Fraction 8.

The partially purified fraction consisted of round and oval granules 0.4 to 1.5 µm in diameter. There was no contamination by mitochondria (Figure 3A). When this sample was stained by the renin-antibody-immunogold technique, all granules were stained posi-
Figure 3. Electron micrograph of subcellular organelles recovered in the partially purified fraction (Fraction 8). A. Renin granules were stained only with uranyl acetate (magnification × 19,000). No contamination of mitochondria can be seen. B. Granules were stained with renin-antibody and gold-labeled goat anti-rabbit IgG (magnification × 55,000).

Discussion

Renin secretion is known to be proportional, to some extent, to the renal renin content, and the content is correlated with the number of renin granules in the juxtaglomerular cells. The storage of renin in renin granules seems to occur in the main pathway to renin secretion. Because renin has been reported to exist in multiple molecular weight forms in male mouse submaxillary gland and kidney, determination of the biochemical nature of renin in renin granules is an approach to the clarification of the renin secretory process.

Although various methods, such as free-flow electrophoresis, a sieving method, and sucrose density gradient centrifugation, have been employed for the isolation of renin granules, these methods have not...
produced completely satisfactory results. In the present study, we applied a Percoll density gradient centrifugation for the isolation of renin granules and obtained a 500-fold purification in specific renin activity and 14% in recovery over the original homogenate. Thus, this method appears to be superior in purity and convenience to the previous methods. There are several reasons for the increased purity of renin granules achieved by this method. First, a primary renin granule fraction (F3) can be obtained quickly. As Percoll has low viscosity, renin granules sediment to an equilibrium position in the gradient in 15 minutes at 40,000 g as compared with 90 minutes at 60,000 g in a sucrose density gradient. Second, the primary granule fraction can be quickly recenterfuged for a further separation by a continuous Percoll density gradient. Third, during the isolating procedures, renin granules are kept in isosmotic conditions. Thus, the prevention of osmotic shock, as well as physical shock resulting from prolonged manipulation of the pellet, reduces damage to the granule during purification.

When the purity of the partially purified renin granule fraction was examined by electron microscopy, all granules were stained positively for renin. No granules were stained in controls using normal rabbit sera. Thus, all granules that were recovered in the fraction seem to contain renin, even in small quantities. The complete purification of renin from rat kidney requires a 6000-fold to 8000-fold purification. In the present work, renin activity was enriched 500-fold in the partially purified renin granules. Thus, it is in agreement with the present finding that approximately 10% of the granules constitute the major storage vesicles of renin. These intensely stained renin granules containing renin at a very high concentration may be considered the "mature" renin granules proposed by Barajas. He has also described progranules (immature granules), which are 0.09 to 0.4 μm in diameter and round or diamond shaped. On the other hand, approximately 90% of the granules seem to contain only small quantities of renin, as indicated by the low density of gold particles in the electron microscopic studies. The present preparation of renin granules contained some lysosomal enzyme activity. Earlier reports indicated the coexistence of renin and lysosomal enzymes in what the authors called "renin granules." Thus, the likelihood that granules containing lower concentrations of renin may have mixed properties of renin granules and lysosomes cannot be eliminated.

The renin in the partially purified fraction from rat kidney was in the active form. On the other hand, we found that inactive renin constitutes 20% of total renin activity (active renin + inactive renin) in human renin granules and 0.3% in dog renin granules. We also found inactive renin in microsomal fractions in rat kidney (unpublished observation). Thus, conversion of inactive to active renin during maturation of renin granules appears to be more rapid in dogs and rats than in humans. The molecular weight of the stored renin showed one peak in the vicinity of 40,000 by gel chromatography. This observation is in agreement with those reported from other laboratories. By sodium dodecyl sulfate gel electrophoresis, however, two different molecular weights (38,000 and 36,000) of renin were found in the granule fraction in the present studies. Purified rat kidney renin was also found to exist in these two molecular weights. In contrast to the active renin, the inactive renin of rat kidney has been shown to have a molecular weight of 48,000 by gel electrophoresis. These results are all in agreement with the concept that the 36,000 and 38,000 (molecular weight) renins in the present renin granule preparation are of the active form.

Pratt et al. indicated that granules of the mouse submandibular gland contain two-chain renin that consists of a heavy chain and a light chain linked by a disulfide bridge, as was found in purified mouse submandibular gland renin. However, the molecular weights of stored renin in rat kidney were not changed by treatment with thiol reductant. These results suggest that the stored renins in the rat kidney are of the one-chain type. We cannot exclude the possibility that renin with a molecular weight of 36,000 could be an artifact generated during the isolation procedures. Further studies are needed to determine whether these two forms of renin are released from the juxtaglomerular cells or from different sources.

In conclusion, the results reported herein indicate that active renin of two different molecular weights is stored in renin granules of the rat kidney and is secreted from mature renin granules.

References

1. Davis JO. Mechanism regulating renin release. Physiol Rev 1956;18:143-157
RAT KIDNEY RENIN GRANULES/Kawamura et al.

The storage form of renin in renin granules from rat kidney cortex.
M Kawamura, J C McKenzie, L H Hoffman, I Tanaka, M Parmentier and T Inagami

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