RENIN PRECURSOR IN HOG KIDNEY. OCCURRENCE AND PARTIAL CHARACTERIZATION

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ABSTRACT

A completely inactive renin precursor was found in hog kidney extract and was separated by two cycles of pepstatin-aminohexyl-agarose or Affi-Gel Blue column from active renin and its active higher molecular weight form ($M_r = 50,000$ – $63,000$). This inactive renin had no affinity for pepstatin, but gained this affinity after trypsin activation. The trypsin activated form of inactive renin was completely inhibited by antibodies raised against pure hog renin. In addition, this inactive renin could also be completely activated by trypsin even after acidification at pH 3.2.

The molecular weight of inactive renin shifted from $43,000 \pm 1,500$ to $41,000 \pm 1,400$ daltons after trypsin activation. After trypsin activation, inactive renin was able to bind the renin binding protein in hog kidney extract to form a high molecular weight renin ($M_r = 60,000$), although it had not been able to do so before activation. These results show that the inactive renin found in the present study is different not only from active renin or acid-activatable form of renin but also from its active higher molecular weight forms found previously by other investigators.

KEY WORDS renin / renin precursor / completely inactive renin / trypsin activatable renin

The only known function of renin (EC 3.4.99.19) is to produce angiotensin I from angiotensinogen. Since renin catalyzes the first step of the renin-angiotensin-aldosterone cascade, the importance of this enzyme in blood pressure regulation and certain types of hypertension is well recognized.

Renin is known to exist in more than one form with different molecular weights in the kidney, plasma, and other tissues (10, 16, 19). The presence of activatable forms of renin has also been reported. Earlier studies on hog and rabbit kidney renin by Boyd (1) and Leckie (14) suggested that the activatable form was in a high molecular weight form ($M_r = 55,000$ – $60,000$) whereas active renin was in a low mo-

lecular weight form ($M_r = 40,000$). Studies by Day and Luetscher (3, 4) of human plasma renin also showed that the activatable form is in a large form ('big renin') whereas the active plasma renin is in a small form. These studies indicated the high molecular weight renin would be the most promising candidate for renin precursor.

However, the high molecular weight renins purified or isolated by various methods from hog (11) and human (20) kidney were found to be already active and failed to show the capacity for activation. Thus, a hypothesis that the high molecular weight renin is the renin precursor or renin zymogen is not supported by clear-cut experimental evidence.

Boyd (1), Leckie and McConnell (14), recently Funakawa *et al.* (7) and we (16, 17) gave ev-

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idence favoring the view that higher molecular weight renin in hog kidney is a complex of lower molecular weight renin and renin binding protein and is not a renin precursor.

On the other hand, the presence of completely inactive renin ($M_r=43,000$ – $55,000$) in hog brain (12), human plasma (21), and amniotic fluid (13) has been reported. The completely inactive renins are activated remarkably by trypsin. They seem to be the renin precursors.

However, there is no report on this type of inactive renin in the kidney, although the kidney is the main production site for renin and is supposed to contain renin precursor.

In the present study, we removed active renin completely from hog kidney extract by an affinity chromatographic method and found a new renin which was both completely inactive and trypsin activatable. This inactive renin with a low molecular weight ($M_r=43,000$) was clearly different from active renin and the active high molecular weight renins ($M_r=55,000$ – $60,000$) found by many investigators (1, 3, 4, 7, 11, 14, 17, 20).

MATERIALS AND METHODS

Chemicals

Agarose coupled with Cibacron Blue F3GA dye (Affi-Gel Blue) was obtained from Bio-Rad. Sepharose-4B was from Pharmacia. Ultrogel AcA 44 was from LKB. Sodium tetrathionate and pepstatin were purchased from Merck and Protein Research Foundation (Osaka, Japan), respectively.

Kidney Extract

Two grams of fresh or frozen hog kidney cortex were homogenized in 8 ml of 0.01 M sodium pyrophosphate buffer, pH 6.5, containing 0.1 M NaCl, for 5 min at 10,000 rpm in an ACE (Nihon Seiki) homogenizer, centrifuged at 100,000 g for 60 min, and the supernatant was used as kidney extract.

Renin Activity

Renin activity was determined by the rate of formation of angiotensin I from hog renin substrate. After incubation with 15 mg of hog angiotensinogen at 37°C for 1 hr in 0.1 M phosphate buffer, pH 6.5, containing 5 mM EDTA and 5.4 mM diisopropyl phosphorofluoridate, angiotensin I was determined by the radioimmunoassay method of Haber *et al.* (8).

Molecular Weight

The apparent molecular weight of renin was estimated by gel filtration of a 1 ml sample on a calibrated column (1.5×90 cm) of Ultrogel AcA 44 (LKB). The column was eluted at a flow rate of 5 ml/hr with 0.01 M pyrophosphate, pH 6.5, containing 0.1 M NaCl. The column was calibrated using the following molecular weight standards: bovine serum albumin ($M_r=67,000$), egg albumin ($M_r=45,000$), and α -chymotrypsinogen A ($M_r=25,000$). All the procedures described above were carried out at 4°C unless otherwise stated.

Affinity Chromatography

The chromatographic properties of inactive renin were determined on pepstatin and Affi-Gel Blue affinity columns as described below. A 2.5×10 cm column of pepstatin-aminohexyl-Sepharose (18) was charged with the kidney extract (40 ml), washed, first with 0.01 M pyrophosphate buffer, pH 6.5, containing 0.1 M NaCl and then with the same buffer containing 1 M NaCl. The column was eluted with 0.5 M Tris buffer, pH 7.5. Fractions of 5 ml were collected at a flow rate of 10 ml/hr. Rechromatography of the non-retained fractions (5 ml) was carried out on a second smaller pepstatin column (1.0×8 cm). The adhering fractions from the first and second pepstatin columns were designated as Peak Ia and Peak Ib, respectively. Inactive renin in Peak Ia was further characterized on an Affi-Gel Blue column (1.0×8 cm). Peak Ia (5 ml) was dialyzed against 0.02 M phosphate buffer, pH 7.1, and applied to the column. After washing the column with the phosphate buffer, inactive renin was eluted with 0.2 M NaCl.

Trypsin Activation

Inactive renin was activated by incubating aliquots (100 μ l) of the chromatographic effluent with 10 μ l of trypsin (1 mg/ml) for 15 min at 37°C. The action was terminated by adding 10 μ l of soybean trypsin inhibitor (10 mg/ml). Controls were treated in the same manner without trypsin.

Anti-renin Antibody

The effect of antibody on the activity of the trypsin activated enzyme was examined by using anti-renin antibody No. 624 which had been raised against pure hog renal renin (9). Acti-

vated renin solutions (100 μ l) were incubated with 10 μ l of the antiserum (1:6 dilution) for 16 hr at 4°C. Residual renin activity was measured by allowing the mixture to react with partially purified hog renin substrate.

RESULTS

Inactive Renin Precursor

A large portion of the active renin present in the kidney extract was removed by absorption on the pepstatin-aminohexyl-Sepharose column. Trypsin treatment of the remainder which

was not absorbed by the affinity column revealed the presence of an inactive form of renin (Fig. 1A). Rechromatography of this material on the second pepstatin affinity column completely removed active renin and showed the presence of entirely inactive renin precursor (Fig. 1B). When the inactive renin precursor (Fig. 1B) was activated by trypsin and applied to the pepstatin column, little or no activity was detected in the non-retained fractions (Fig. 1C). Upon elution with 0.5 M Tris buffer, pH 7.5, however, a single peak of renin activity appeared, indicating that the inactive renin precursor was converted to the active enzyme, which had been shown to have affinity for this column. The angiotensin I forming activities of both the active and the trypsin-activated product were completely neutralized by anti-renin antibody (Table 1, a and b).

Fig. 1 Separation of active and inactive renin in hog kidney extract by affinity chromatography on a pepstatin-aminohexyl-Sepharose column. Samples applied to the column were: A, 40 ml of hog kidney cortical extract; B, 5 ml of the non-retained fraction in experiment A (Peak Ia); C, 5 ml of the non-retained fraction in experiment A (Peak Ia) after trypsin activation; and D, 5 ml of Peak Ia after acidification. Fractions after the affinity chromatography were also activated by trypsin: —●—, renin activity before activation; —○—, renin activity after trypsin activation. The symbol —●— shows that the renin activity before activation is the same as that after trypsin activation. The other experimental conditions are described in Materials and Methods. Ang I = Angiotensin I

Acidification of Inactive Renin

The acidification of inactive renin at pH 3.2 resulted in appreciable activation of inactive renin, though it was much less extensive compared with activation by trypsin (Table 1, a, b and c). Furthermore, the acidified inactive renin was activated by trypsin to the same level as the non-acidified materials (Table 1). This result seems to rule out the possibility that the inactive renin was a complex of active renin and an acid-labile inhibitor, which is known to be dissociated by the acid treatment. The chromatographic properties of the inactive renin on the pepstatin affinity column were not altered after acidification. Acidified inactive renin (Fig. 1D) was eluted in early effluent fraction where untreated inactive renin (Fig. 1B) was expected, and remained inactive until being treated with trypsin.

Shifting of the Molecular Weight of Inactive Renin upon Activation

The molecular weight of inactive renin and active renin were estimated by gel filtration chromatography as $43,000 \pm 1,500$ (N=5) and $41,000 \pm 1,400$ (N=8), respectively. Although the difference in molecular weight was small, it was significant. Run-to-run variation was small and the elution patterns were highly reproducible. Chromatography of a mixture of inactive and active renin on the Ultrogel column to determine the difference in molecular weight confirmed these results by giving a molecular weight of 43,000 for the inactive precursor and 41,000 for active renin (Fig. 2).

Table 1 *Effect of Anti-renin Antibody on Inactive Renin and Its Activated Form*

	Inactive renin* ¹	Trypsinization* ²	Acidification* ²	Renin activity (ng Ang I/ml enz/hr)	
				—Antibody	+Antibody* ³
a	+	—	—	0.10	0.01
b	+	+	—	9.50	0.73
c	+	—	+	0.32	0.01
d* ⁴	+	+	+	10.75	1.03

*¹ The non-retained fraction in the second pepstatin affinity column (Peak Ib in Fig. 1) was diluted 10 times and used as inactive renin.

*² A 100 μ l aliquot of the inactive renin was trypsinized and acidified as described in Materials and Methods.

*³ Incubation of the inactive renin with antibody was also carried out as described in Materials and Methods.

*⁴ Inactive renin was acidified and then trypsinized.

Fig. 2 Gel filtration of a mixture of active and inactive renins on a calibrated Ultrogel AcA 44 column. One ml of the non-retained fraction in the first pepstatin affinity column (Peak Ia in Fig. 1A) was acidified and applied to the Ultrogel AcA column. This acidified fraction contained both active and inactive renins although it had no renin binding protein and could not catalyze interconversion between active renin and its higher molecular weight form (17). The column was developed and fractions after gel filtration were activated by trypsin as described in Materials and Methods. —●—, renin activity before activation; —○—, renin activity after trypsin activation; ●, standard proteins; ○, active and inactive renins.

Distinction of Inactive Renin from Active Renin by Renin Binding Protein

The presence of a renin binding protein has been reported (1, 7, 14, 17) in the hog and dog kidney. When incubated with Na-tetrathionate, a thiol blocker, active renin was converted to a high molecular weight form in the hog kidney extract (—●—, Fig. 3A and B). These bindings were reversible. In contrast to active renin, incubation of inactive renin with the binding protein and Na-tetrathionate showed no molec-

ular weight shift (—○—, Fig. 3A and B). After trypsin activation, however, it showed binding to the binding protein as indicated by a marked shift in molecular weight to the high molecular weight form (Fig. 3C). Acidification of this complex converted it to the low molecular form, presumably due to dissociation of the complex.

Binding of Inactive Renin to Affi-Gel Blue

Inactive renin in human plasma (21) and amniotic fluid (13) has been found to bind to Cibacron Blue F3GA-agarose (Affi-Gel Blue) column, whereas active renin does not bind to this gel. The inactive renin in the hog kidney was found to be bound to the Affi-Gel Blue column and eluted with 0.2 M NaCl, as expected for plasma inactive renin, while the active hog kidney renin passed through the column and appeared in non-retained fractions. This clear separation is demonstrated in Fig. 4.

DISCUSSION

A number of reports (19) have been published on acid activation of renin and the possible existence of inactive renin or renin precursor in the kidney. However, these reports have been controversial. The proportion of active and inactive renin in kidney was found to depend on many factors such as species, pathophysiological conditions, and extraction method (19).

The presence of completely inactive renin was demonstrated in human plasma (21). However, direct evidence for its presence in its source organ has not been available. A number of investigators have reported acid activation of renin activity in kidney extracts (see for ex-

Fig. 3 Interaction between renins and renin binding protein examined on an Ultrogel AcA 44 column (1.6×90 cm). The non-retained fraction on the first pepstatin affinity column (Peak Ia in Fig. 1A) was used for the sample applied to the column, because it contained a suitable amount of active and inactive renins and renin binding protein. Before application to each column, a 1 ml sample was incubated in the following way: A, at 4°C for 2 days; B, at 37°C for 15 min in the presence of 5 mM Na-tetrathionate; and C, with $4.2 \mu\text{M}$ trypsin (0.1 mg/ml) at 37°C for 15 min followed by additional incubation with 5 mM Na-tetrathionate. The symbols in Fig. 3 are the same as in Fig. 1. The other experimental conditions are described in Materials and Methods.

Fig. 4 Affi-Gel Blue column chromatography of the active and inactive renins. The non-retained fraction on the first pepstatin affinity column (Peak Ia in Fig. 1A) was applied to Affi-Gel Blue column and eluted as described in Materials and Methods. The symbols in Fig. 4 are the same as in Fig. 1.

ample a review by Overturf, 19), suggesting the presence of an activatable form of renin in this

tissue. However, it has not been clear whether the activatable form was a partially active enzyme with a potential for further activation, an enzyme-inhibitor complex or a totally inactive precursor.

The separation of the totally inactive enzyme from active enzyme by two steps of affinity chromatography and subsequent activation by trypsin clearly demonstrate the presence of a completely inactive renin precursor in the kidney (Fig. 1B). Although the finding of inactive renin in the plasma of anephric humans indicates that some extrarenal tissues have the capability to synthesize inactive renin precursor, the present finding indicates that the kidney is also a source of renin precursor. Possible contribution of inactive renin from plasma was eliminated by exhaustive perfusion of hog kidneys immediately after killing of the animal.

Furthermore, we (Murakami K. *et al.* unpublished data) found that hog plasma obtained from the jugular vein at a slaughterhouse contained a total renin activity of, at most, 0.7 ng angiotensin I/mg plasma protein/hr after trypsin activation. This is one-tenth the concentration of inactive renin present in the kidney extract (7 ng angiotensin I/mg protein/hr; Fig. 1A).

The facts (Table 1 and Fig. 1) that the active enzyme derived from the inactive precursor by trypsin treatment is inactivated by specific anti-renin antibody, that the activated enzyme is bound to renin binding protein, and that it elutes from the pepstatin column in a manner identical with active renin are in agreement with the notion that the inactive form isolated in the present study is renin precursor.

The proportion of inactive to active renin in the hog kidney was found to be less than 1% (Fig. 1A). Almost the same inactive/active ratios were found in kidneys of rats and humans (Murakami K. *et al.* unpublished data). On the other hand, plasma has 40–70% of its renin activity in the form of inactive renin (2, 15). An explanation for this apparent discrepancy in the percentage of inactive renin could be that the clearance rate of active renin in circulation is much higher than that of inactive renin. This difference in the clearance rate results in the accumulation of inactive renin with a longer half-life. In fact, Derkx *et al.* (5) reported that a) both active and inactive renin fell rapidly after bilateral nephrectomy in 12 patients and b) estimated that the half-life in two patients was 30–80 min for active renin and 150–165 min for inactive renin.

Since it is believed that the blue dye Cibacron Blue F3GA binds to relatively hydrophobic regions in proteins that recognize nucleotides (6), it seems reasonable that the difference between active and inactive renin in the affinity for Affi-Gel Blue column (Fig. 4) represents the difference in hydrophobicity. Whether the inactive renin might have a nucleotide-binding site remains to be elucidated.

In inactive renin, both the active site and the binding site for renin binding protein seem to be blocked or altered in such a way that angiotensinogen, pepstatin, and the binding protein are no longer capable of interacting with the enzyme. The activation of inactive renin by trypsin must somehow alter the molecule so that the active site and the binding site become exposed. This is because the activated renin gains an ability to be completely retained on pepstatin affinity column and to bind the binding protein. However, it should be noted that the inactive renin before the activation does not do so. Upon converting inactive renin to active renin, small peptide fragment (or fragments) is probable to be lost although it was not possible to estimate exactly the molecular weight of this peptide(s) by gel filtration. Our result concerning a small molecular weight shift upon the trypsin activation is in agreement with observations on inactive renins in human amniotic fluid (13), hog brain renin (12), and human plasma (21). It appears that inactive renin precursor has no inherent enzymatic activity but is truly an inactive form of renin which only shows enzymatic activity after the appropriate activation has occurred.

It is known that higher molecular weight renin ($M_r=50,000-60,000$) in the kidney is converted to low molecular weight renin ($M_r=40,000$) by acidification and is accompanied by a fairly big molecular weight shift (1, 14, 19). Thus this molecular shift caused by acidification is different from the very small one observed in the trypsin activation of inactive renin. Moreover, inactive renin was neither activated nor destroyed by acidification. These results show that the mechanism of acid activation of renin in the kidney is not the same as that of the trypsin activation of inactive renin.

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