INTERMEDIATE MOLECULAR WEIGHT RENIN AND RENIN-BINDING PROTEIN(S) IN THE HOG KIDNEY

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ABSTRACT

The pressor enzyme renin (EC 3.4.99.19) exists in a high molecular weight ($M_r=60,000$) and a low molecular weight form ($M_r=40,000$) in kidney extracts. However, the mechanism responsible for the conversion between the two forms of renin is unclear.

We found a new form of renin which was intermediate between the high molecular and the low molecular weight renin in kidney extract. The incubation of hog kidney extract at 37°C for 60 min yielded a renin of intermediate size ($M_r=50,000$) from low molecular weight renin. This new form was converted to the high molecular weight form by the addition of sodium tetrathionate, a sulfhydryl reagent. The reverse conversions, *i.e.*, from the high molecular to the intermediate size and then from the intermediate to the low molecular weight form were mediated by dithiothreitol and acid or alkali treatment, respectively. We further demonstrated the presence of renin-binding substance(s) in the hog renal cortex and subsequently separated them completely from renin by a pepstatin-aminohexyl-agarose column. When incubated with a preparation of this binding substance, pure hog renal renin ($M_r=40,000$) was converted to the high molecular weight form through the intermediate form. This conversion could also be reversed. These results indicate that the intermediate molecular and the high molecular weight renin is the reversible complex of lower molecular weight renin and the renin-binding substance(s). Furthermore sulfhydryl group(s) of renin and/or the binding substance(s) are a key factor in the dissociation and association of the complex.

KEY WORDS renin / intermediate molecular weight renin / renin-binding protein

The enzyme renin (EC 3. 4. 99. 19) catalyzes the formation of the decapeptide angiotensin I from angiotensinogen and plays a key role in blood pressure regulation. Pure renin preparations isolated from the kidney of hog (3, 13, 24), rat (22), dog (7) and man (9, 34, 35) have been shown to have molecular weights ranging from 35,000 to 40,000. In recent years the existence of high

molecular weight forms of renin ($M_r=55,000-63,000$) have been reported in the kidney, plasma and amniotic fluid in various mammals (2, 4, 5, 12, 14, 16, 19, 20, 21, 31, 33). The proportion of the high molecular to low molecular weight form was found to vary extensively depending on tissues, mammalian species and disease states (28).

In our previous study (15) we reported that extraction of hog and rat kidneys in the presence of blockers of sulfhydryl (SH) groups, sodium tetrathionate or *N*-ethylmaleimide, produces

exclusively high molecular weight renin. We also proposed that the native form of renin in the kidney is the high molecular weight form and that it is converted by an enzyme or agent requiring SH groups to the low molecular weight (circulating) form.

However, little is known concerning the mechanism of the conversion of renin between the two forms. In this report, we observed that the conversion between the two forms of renin is reversible and this reversible conversion proceeds through an intermediate molecular weight form of renin ($M_r=50,000$). Our objective in the present study is to clarify the conditions and mechanism which determine the interconversion of the high, intermediate and low molecular weight forms of hog renin.

MATERIALS AND METHODS

Chemicals

Bovine serum albumin (BSA) and soybean trypsin inhibitor were obtained from Seikagaku Kogyo. *N*-Ethymaleimide was from Fluka. Sodium tetrathionate was from Merck. Ultrogel AcA 44 was from LKB. Ovalbumin, α -chymotrypsinogen and diazenedicarboxylic acid *bis* (dimethylamide) (diamine) were from Sigma. Iodoacetic acid, iodoacetamide, dithiothreitol and 5,5'-dithio *bis* (2-nitrobenzoic acid) (DTNB) were all purchased from Wako Chemicals. Pepstatin, chymostatin and leupeptin were from Protein Research Foundation. *Trans*-epoxy-succinic acid inhibitor, E-257, was a gift from Dr K. Hanada.

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 renin substrate which had been obtained from hog plasma as described below. After incubation with 15 mg of hog angiotensinogen preparation (see below) 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.* (10).

Hog Renin Substrate

Two liters of fresh blood obtained from normal hog in 0.3% EDTA was centrifuged at 3,400 *g* for 30 min. The pH of the supernatant was adjusted to 5.3 and solid ammonium sulfate was added to a concentration of 30.4 g per 100 ml. After standing for 1 hr, the resulting precipitate was separated by centrifugation, suspended in 200 ml of distilled water and dialyzed for 12 hr against 0.01 M acetate buffer pH 5.5. The dialyzed sample was centrifuged at 9,000 *g* for 20 min. The supernatant was applied to a pepstatin-aminoethyl-agarose column (13, 24) (1.5 $\times$ 4.5 cm) equilibrated with 0.01 M acetate, pH 5.5, to remove renin and eluted with the same buffer at a flow rate of 60 ml/hr. Renin substrate fraction containing almost no renin activity was eluted in the breakthrough fractions. The renin substrate in the eluate was again salted out by the addition of solid ammonium sulfate as described above. The resulting precipitate was dissolved in 200 ml of distilled water and dialyzed for 12 hr against distilled water. The dialyzed sample was centrifuged and the supernatant was used as hog angiotensinogen preparation after lyophilization. Fifteen mg of the hog renin substrate was added to each renin assay tube to give maximum amount of angiotensin I during incubation.

Molecular Weight

The apparent molecular weight of renin was estimated by gel filtration of a 1 ml sample on a calibrated column (1.5 $\times$ 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.

Acid or Alkali Treatment

The pH of the extracts or fractions containing renins was lowered to 3.0 or raised to 10.5 with HCl or NaOH and neutralized immediately after 1 min standing at 4°C.

Modification of SH-Groups

To examine the effect of sulfhydryl reagent on the interaction between renin and renin-binding substance(s), hog kidney extracts (1 ml) were treated with two types of reagent; SH-oxidizing (Na-tetrathionate, DTNB, and diamine) and SH-alkylating reagents (*N*-ethylmaleimide, iodoacetic acid and iodoacetamide). Experimental conditions and the concentration of various modification reagents are included in Table 2.

Isoelectric Focusing

Isoelectric focusing in density gradients (32) was performed at 5°C in a 110 ml glass column (Kato Co.) in 1% ampholyte solutions. The pH was measured at 4°C using a Beckman Expandomatic SS-2 pH Meter.

Pure Renin

Pure renin was isolated from hog kidney extract by a published procedure (13, 24) consisting of affinity chromatography on a pepstatin-aminohexyl-agarose, gel-filtration on Sephadex G-100 and chromatography on DEAE-cellulose. The renin preparation after DEAE-cellulose chromatography already had a very high specific activity almost comparable with that of pure renin, and showed a single band on polyacrylamide gel electrophoresis at pH 8.9. It was used as a pure renin in the present investigation.

Preparation of Renin-Binding Protein

The hog kidney extract (40 ml) as described above was applied to pepstatin-aminohexyl-agarose column (1.5 × 10 cm) at a flow rate of 10 ml/hr. The breakthrough fraction (33 ml) of the eluate containing no renin was collected, mixed and used as a preparation of renin-binding protein.

RESULTS

Interconversion between High Molecular and Low Molecular Weight Renin in Kidney Extract

Extract of both fresh or frozen hog kidney cortex prepared in the absence of an SH-blocker was found to contain the low molecular weight form of renin ($M_r = 40,000 \pm 2,000$ S.D., $n = 11$) as examined by gel filtration on Ultrogel AcA 44. Addition of the SH-blocker, Na-tetrathionate (10 mM) to the extraction buffer shifted the renin

activity from the low molecular weight fractions to the high molecular weight fractions ($M_r = 60,000 \pm 2,500$ S.D., $n = 7$).

Although these observations are in agreement with our postulate that a sulfhydryl enzyme in the kidney extract causes the breakdown of the high molecular weight renin to low molecular weight renin (15), it is also possible that the conversion represents the dissociation of a complex of renin and renin-binding substance or the association of both substances.

To test this possibility, hog kidney extract containing only the low molecular weight enzyme (Fig. 1A) was incubated with 5 mM Na-tetrathionate at 37°C for 15 min. Gel filtration of this mixture showed that low molecular weight renin was converted almost completely to the high molecular weight form (Fig. 1C).

Treatment of high molecular weight renin by acid is known to reduce it to the low molecular weight form. The above solution converted to the high molecular weight form (represented by Fig. 1C) was subjected to the acid treatment at pH 3.0. Only low molecular weight renin was found upon gel filtration.

Fig. 1 Gel filtration on an Ultrogel AcA 44 column (1.5 × 30 cm) of hog kidney renins. A) Crude extract without reagent. B) Incubation of C at 37°C for 15 min with 50 mM dithiothreitol. C) Incubation of A at 37°C for 15 min with 5 mM Na-tetrathionate. Ang I = Angiotensin I

Intermediate Molecular Weight Renin

Results reported above indicated the possibility of the reversible formation of high molecular weight renin from low molecular weight renin and a small protein fragment or fragments through a disulfide bridge formation catalyzed by sodium tetrathionate.

To examine this hypothesis, an extract containing the high molecular weight renin induced by sodium tetrathionate was incubated with a large excess of dithiothreitol (50 mM) at 37°C for 30 min. Gel filtration of this mixture showed almost complete absence of renin activity in fractions corresponding to the high or low molecular weight enzyme. Instead, a major activity was eluted in intermediate fractions corresponding to an apparent molecular weight of $49,000 \pm 1,000$ S.D. ($n=10$) as shown in Fig. 1B.

To investigate the reversibility of the conversion of intermediate molecular weight renin to high molecular weight renin, the mixture containing this new form of renin and dithiothreitol was dialyzed for 20 hr against 0.01 M pyrophosphate, pH 6.5, containing 0.1 M NaCl to remove dithiothreitol, then it was incubated with 10 mM sodium tetrathionate at 37°C for 15 min. Examination by gel filtration indicated that the intermediate molecular weight renin was converted back to the high molecular weight form. The acid treatment of the intermediate molecular weight renin produced low molecular weight renin. These results indicate that the conversion of high molecular weight renin to low molecular form and back proceeds through the intermediate form.

The intermediate renin was also formed when kidney extract containing the low molecular weight renin was incubated at 37°C for 60 min or at 4°C for 1 week. Even at -20°C without any additional reagent, slow formation of this form of renin was also noted. Low molecular

weight renin decreased gradually and proportionally to the increase of intermediate molecular weight renin. The molecular weight of intermediate molecular weight renin was estimated to be $49,500 \pm 1,800$ S.D. ($n=6$). Whether the intermediate form of renin formed in the absence of dithiothreitol is identical with that formed in the presence of this reagent is not known since we were working with crude kidney extracts.

Table 1 shows the enzyme activity and some properties of the three forms of renin. Low molecular weight renin in hog kidney extract prepared at pH 6.5 without the tetrathionate had the highest activity. The intermediate molecular and high molecular weight renins had activities significantly lower than that of the low molecular weight enzyme. Upon acid treatment at pH 3.0, the low molecular weight renin lost its activity by 13%. The intermediate and the high molecular weight renins increased their activity under acid treatment with concomitant molecular shift to the low molecular form.

Effect of SH-Reagents on the Reversible Conversion

Two experiments were conducted to investigate the nature of the involvement of the sulfhydryl group in the interconversion. First, Na-tetrathionate (23), DTNB (23) and diamide (18) were used to oxidize sulfhydryl groups to disulfide groups. This reaction can be reversed by dithiothreitol. Second, *N*-ethylmaleimide, iodoacetic acid and iodoacetamide were used to alkylate sulfhydryl groups in an irreversible manner.

These experiments are summarized in Table 2. All the reversible and irreversible reagents effected complete conversion of low molecular weight renin to the high molecular weight form in 30 min at 4°C or in 15 to 30 min at 37°C. Intermediate molecular weight renin was formed by treatment with dithiothreitol of high molecular

Table 1 Properties and Activities of Three Forms of Renin

Molecular form of renin	Molecular weight	Isoelectric point	Renin activity ^c	
			Before acidification	After acidification
High	$60,000 \pm 2,500$ ($n=7$)	5.30 - 5.36	9.84 ± 0.06	12.71 ± 1.24
Intermediate-1 ^a	$49,500 \pm 1,800$ ($n=6$)	5.24 - 5.34	10.78 ± 0.57	15.88 ± 1.27
Intermediate-2 ^b	$50,500 \pm 1,900$ ($n=5$)	5.28 - 5.30	9.30 ± 0.40	13.68 ± 0.60
Low	$40,000 \pm 2,000$ ($n=11$)	5.35 - 5.38	17.12 ± 1.48	15.17 ± 1.01

^a Incubation of low molecular weight renin at 37°C, 60 min

^b Incubation of high molecular weight renin at 37°C, 30 min with 50 mM dithiothreitol

^c μ g Angiotensin I/ml of kidney extract/hr, number of experiment ($n=5$)

Table 2 *Effect of SH-Reagent on the Interconversion among Three Forms of Renin*

Reagent	Conditions			Molecular weight of renin ($\times 1,000$)
	Concentration (mM)	Temperature ($^{\circ}\text{C}$)	Time (min)	
None	—	4	30	40
None	—	37	60	50
SH-oxidizing reagents				
1) Na-tetrathionate	5	37	15	60
2) DTNB	10	4	30	60
3) Diamide	5	37	5	60
4) 1, 2 or 3)+dithiothreitol	50	37	30	50
SH-alkylating reagents				
5) <i>N</i> -ethylmaleimide	10	4	30	60
6) ICH_2COOH	10	37	15	60
7) $\text{ICH}_2\text{CONH}_2$	10	37	15	60
8) 5, 6 or 7)+dithiothreitol	50	37	30	60

weight renin induced by reversible oxidants (No. 4, Table 2). As expected, the same treatment failed to produce the intermediate form if the high molecular weight renin had been formed by the alkylating reagents which modified SH-groups irreversibly (No. 8, Table 2).

Reconstitution of High Molecular and Intermediate Molecular Weight Renin from Low Molecular Weight Renin and Renin-Binding Protein Fraction

To obtain definitive evidence for reversible conversion among the three forms of renin in kidney extract, we investigated the reaction of completely purified low molecular weight renin with renin-binding protein fractions. Pure low molecular weight renin ($1\text{ }\mu\text{g}$) was completely converted to intermediate molecular weight renin ($M_r = 50,000$) upon incubation with 1 ml of the renin-binding protein fraction at 37°C for 60 min. The intermediate form was further converted to the high molecular weight form ($M_r = 60,000$) by an additional incubation of the mixture with 5 mM sodium tetrathionate at 37°C for 15 min.

In reversal of these reactions, incubation of the high molecular weight renin with 50 mM dithiothreitol converted it to the intermediate

form, which was further converted to low molecular weight renin by acid treatment at pH 3.0 under the conditions described earlier.

High molecular weight renin in kidney extract or reconstituted as above was also converted directly to low molecular weight renin by acid (pH 3.0) or alkali (pH 10.5) treatment.

However, the reversible nature of these reactions was totally lost following the acid or alkali treatment. This was because of the destruction of the binding protein. The low molecular weight renin itself still retained the ability to react with a freshly added binding protein fraction. These characteristics of renin and the binding protein were similar to those reported by Boyd (2) for hog kidney, by Leckie and McConnell (19) for rabbit kidney and by Kawamura *et al.* (17) for dog kidney. An interrelation among high, intermediate and low molecular weight renin is shown in Fig. 2.

The possible involvement of proteases was examined in preliminary experiments in which a mixture of protease inhibitors was added to the interconversion reaction mixture. This inhibitor mixture consisted of 5 mM EDTA, 0.5 mM diisopropyl phosphorofluoridate, soybean trypsin inhibitor (0.5 mg/ml), 50 μM pepstatin (1), 5 mM chymostatin (1), 50 mM leupeptin (1), and 50 mM *trans*-epoxysuccinic acid inhibitor,

Fig. 2

E-257 (11). None of the steps of the reversible or irreversible interconversion was affected by the protease inhibitors.

DISCUSSION

Since the original reports of the existence of activatable form of renin in the crude kidney extract by Rubin (30), Boyd (2) and Leckie (19), controversy has persisted concerning the nature of the various forms of renin and the mechanism of interconversion or activation. Boyd (2) reported the presence of a protein bound form of renin in hog renal extract with a gel filtration molecular weight of 60,000. Treatment with 4 M NaCl was found to dissociate it reversibly to a free form with a molecular weight of 40,000 and a binding protein (2). Leckie and McConnell (19) found that rabbit renal extract contains a partially active form of renin with a molecular weight of 55,000, which can be converted to a low molecular weight form ($M_r=37,000$) by a passage through an ion exchange column. A small inhibitor protein was separated which can recombine with the active form of renin to reduce its activity in a reversible manner. However, subsequent studies on hog kidney by Levine *et al.* (20, 21) and by us (unpublished data) or on human plasma by Day *et al.* (6) failed to reproduce the reversible activation and molecular weight changes by the above mentioned treatment.

Later, we (15) found that use of protease inhibitors, particularly SH-blockers, preserved the high molecular weight form of renin in crude extracts of hog and rat kidney. This finding has been confirmed by Slater and Haber in human kidney extract (31) and by Potter *et al.* (29) in dog kidney renin. These investigators have also shown that omission of the inhibitors results in the recovery of low molecular weight renin. However, it has not been possible to demonstrate reversible interconversion nor appreciable activation and inactivation. Neither has it been possible to isolate the inhibitor protein or the binding protein.

In the present studies we approached this problem from the opposite direction using low molecular weight renin and looked for a reversible binding protein or proteins. Using both crude and purified low molecular weight renin from hog kidney, we could demonstrate the presence of such a reversibly binding protein in the soluble fraction of hog kidney cortex. This protein was found to bind renin from other mammals as well (25). Thus, the binding pro-

tein seems to have general affinity for renin from other mammals.

Use of the new gel filtration matrix, Ultrogel AcA 44 permitted us to resolve an intermediate molecular weight renin with an approximate molecular weight of 50,000 from the high molecular weight and low molecular weight form. Using this method, reversible conversion of the high molecular weight form ($M_r=60,000$) to the intermediate form by dithiothreitol was demonstrated. Incubation of renal cortical extract with the intermediate form in the presence of Na-tetrathionate regenerates the high molecular weight form.

These studies indicate that a renin-binding protein or proteins exist in the renal cortical extract and it undergoes reversible binding with low molecular weight form and the intermediate form of renin. Whether the binding protein is an entity independent of renin precursor and serving for regulatory purpose or it is a part of the precursor which has been split off during the process of activation of the precursor to the active enzyme is an interesting problem. Very recently, we (27) found a renin precursor which is completely inactive in native state and remarkably activated by trypsin. This inactive renin precursor is a different substance from the active intermediate and high molecular weight renin reported in the present investigation. Therefore, the binding protein seemed to be a regulatory protein or a carrier protein, but not a part of precursor.

The reversible conversion of intermediate molecular weight renin to the high molecular form was apparently mediated by the reversible modification of SH-group of renin and/or renin-binding protein. This result indicated the SH-group of renin and/or the binding protein is a key factor in their association and dissociation. However, the high molecular weight renin induced by the irreversible modification of the SH-group could not be converted to the intermediate form. This result eliminated a possibility of formation of disulfide-linked complex between renin and the binding protein. We speculated that the modification of SH-group of renin and the binding protein markedly enhances their affinity for each other as reflected in an appearance of the high molecular form. Although we have no direct information on the nature of the chemical bond between renin and the binding protein, the bond may not be covalent. Because the complex of renin and the binding protein easily dissociates into their components at pH 3.0 or pH 10.5. Confirma-

tion of this speculation would be provided after further purification of the binding proteins.

Funakawa *et al.* (8) have shown that renin stored in the renin granules of dog kidney is the low molecular weight form and is capable of binding with a renin-binding substance in the soluble fractions of renal cortical extract. If this result is true in kidneys of all mammals, an interesting question how and where the low molecular weight renin in the granule is converted to the high molecular form, remains to be solved. Day *et al.* (5) reported the presence of 'big renin' in plasma or kidney of patients with renal diseases such as Wilms' tumor and diabetic nephropathy. Recently, we (26) found that the major form of renin in the plasma of the malignant phase of the stroke prone spontaneously hypertensive rats was a high molecular weight renin instead of normal circulating renin of low molecular weight. Although physiological significance of high molecular weight renin is not known, the conversion of low molecular weight renin to the high molecular form *in vivo* may be related with disease conditions.

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