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EVIDENCE FOR A COMPLETELY INACTIVE RENIN ZYMOGEN IN THE KIDNEY BY AFFINITY CHROMATOGRAPHIC ISOLATION

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SUMMARY

A complete inactive renin was isolated from hog kidney extract by hydrophobic interaction chromatography on Octyl-Sepharose followed by affinity chromatography on pestatin-aminohexyl Sepharose. The inactive renin was activated by trypsin. It has a molecular weight of 53,000 ± 2,000 by gel filtration on Sephadex G-100. The active renin generated from this inactive renin by trypsin treatment has the molecular weight of 41,000 ± 1,000. Treatments of the inactive renin with dithiothreitol, NaCl, SDS or an acidic buffer did not cause activation indicating that the inactive preparation is not a renin-inhibitor complex.

INTRODUCTION

In spite of numerous reports on the presence of the completely inactive renin zymogen in the plasma, evidence for such a precursor in its source organ, kidney, has remained elusive. While Haas et al (1) and Rubin (2) have observed that acidification of fresh renal extracts results in an increased yield of renin activity, Leckie (3) and Boyd (4) have presented evidence which suggests that such activation may be due to reversible dissociation of a renin-inhibitor complex which has a molecular weight considerably larger than active renin. Recent studies by Funakawa et al. (5) and Murakami et al. (6) identified the presence of a renin binding protein in the kidney and showed reversible but incomplete inhibition by this protein. While partially (7,8) or completely purified (9) high molecular weight renin isolated from hog and human kidneys were already active and did not show capacity for appreciable activation, it is also a complex of active renin and other proteins. These results seem to suggest that high molecular weight renin in the kidney is a complex of active

Abbreviations: NEM, N-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropylphosphorofluoridate.

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renin and an inhibitor. We have devised a rapid method to separate inactive renin from hog kidney extract by an affinity chromatographic procedure and have obtained evidence suggesting the presence of a completely inactive renin zymogen which does not undergo activation by known dissociative procedures.

METHODS

Kidney extraction Kidneys were excised from pentothal-anesthesized pigs and immediately washed by gentle infusion of ice-cold saline solution (150 ml) into the renal artery. The cortex (40 g) was sliced and homogenized in a Warning blender at 4°C with 200 ml of 0.01 M sodium pyrophosphate buffer, pH 6.6, containing 0.1 M NaCl and the following protease inhibitors: EDTA, 10 m M; NEM, 10 m M; PMSF, 2 m M and DFP, 0.1 m M. The homogenate was centrifuged at 10,000 x g for 20 min at 4°C and the supernatant was retained. The supernatant was fractionated by preciptation with ammonium sulfate. A cut between 1.0 M and 2.0 M ammonium sulfate was collected by centrifugation. Precipitates were dissolved in an equal volume of 0.01 M phosphate buffer, pH 6.9. This solution in approximately 1 M ammonium sulfate was purified further by 2 steps of affinity chromatography.

Affinity chromatography Octyl-Sepharose CL-4B (Pharmacia) (200 ml) was washed on a glass filter funnel in succession with ethanol (200 ml), n-butanol (400 ml), ethanol (200 ml), deionized water (200 ml), and 0.01 M phosphate buffer, pH 6.9 (400 ml) and poured into a 2.5 x 24 cm column. It was equilibrated with 0.01 M phosphate buffer, pH 6.9, containing 1.0 M ammonium sulfate. After application of 20 ml of the extract in 1.0 M ammonium sulfate (prepared from 40g kidney cortex), the column was eluted with the phosphate buffer with stepwise changes in NaCl concentration (4 M, 3 M, 2 M, 1 M and 0 M) and finally with 50% ethylene glycol. The renin containing fractions were concentrated by pressure filtration through an Amicon PM-10 filter, equilbrated with 0.01 M pyrophosphate buffer, pH 6.6, centrifuged to remove precipitates, and applied to a 2.4 x 7.5 cm column of pepstatin-aminohexyl Sepharose (10), previously equilibrated with the same pyrophosphate buffer. After exhaustive wash with the same buffer, active renin was eluted with 0.5 M Tris-HCl, pH 7.5, containing 0.1 M NaCl.

Analytical gel filtration A Sephadex G-100 column (2.5 x 90 cm) was used to estimate the molecular weight of renin. The column was eluted with 0.01 M pyrophosphate buffer, pH 6.6, containing 0.1 M NaCl. Fractions of 1.9 ml were collected. Other experimental conditions were the same as described previously (11).

Renin activity was determined by the raioimmunoassay of angiotensin I (12) generated during incubation for 60 min at 37° of a mixture consisting of 25 µl of the fractionated sample and 25 µl of the purified sheep plasma substrate (11), 200 µl of 0.2 M maleate buffer, pH 6.0, containing 10 µl of 0.2 M EDTA and 5 µl of 0.15 M PMSF.

Activation of inactive renin was obtained using trypsin (Worthington, trypsin-TPCK). To 25 µl of a fractionated solution was added 25 µl of a freshly mixed solution of 2.5 µg of trypsin, 250 µg of bovine serum albumin in 0.1 M Tris-HCl buffer, pH 7.5. After reaction for 30 min at 25°C, trypsin was neutralized by the addition of 40-fold molar excess (100 µg) of soybean trypsin inhibitor.

Anti-hog renin antibody Specific rabbit anti-hog renin antibody (13) was raised against pure hog renal renin (14). Effect of the specific antibody on renin activity was determined after incubating 50 µl of the fractionated renin with 25 µl of diluted antibody (1:10) for 16 hrs at 4°C.

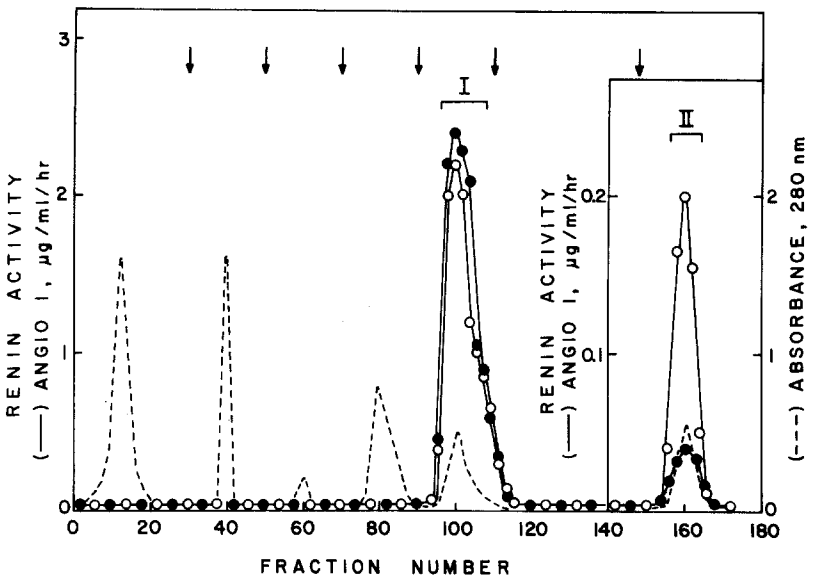

Fig. 1 Hydrophobic interaction chromatography of hog kidney extract on an octyl-Sepharose. Elution of the column was begun with 4 M NaCl in 0.01 M phosphate buffer, pH 6.9 at the position indicated by the first arrow followed by stepwise changes in NaCl concentration to 3, 2, 1 and 0 M as indicated by subsequent arrows, and finally by 50% ethylene glycol in the same buffer. Renin activity was determined before (●) and after (○) activation by trypsin. (---), absorbance at 280 nm.

RESULTS

Isolation of inactive renin As shown in Figure 1 we were able to separate inactive renin from the extract of hog kidney by hydrophobic interaction chromatography on a column of octyl-Sepharose. After unbound proteins were washed out, renin activity was eluted in two separate peaks. The first peak (Peak I-1, Fig. 1) fractions eluted by 1.0 M NaCl accounted for approximately 80% of the total renin activity applied to the column. The second renin peak (Peak II-1, Fig. 1) eluted by 50% ethylene glycol accounted for a very small portion (ca. 15%) of the total renin activity. The peak II-1 materials were combined and fractionated further by affinity chromatography on the column of pepstatin-aminohexyl Sepharose. The non-retained fractions did not contain renin activity beyond the control level (peak I-2, Fig. 2), but the trypsin treatment of the fractions resulted in extensive activation indicating the presence of inactive renin zymogen in those fractions. The newly generated enzyme activity

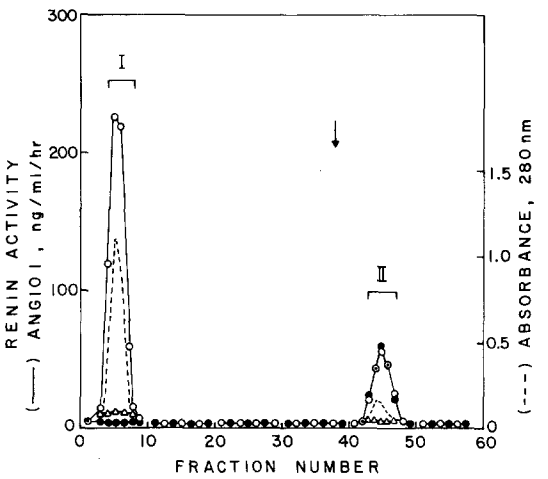

Fig. 2 Affinity chromatography of renin on a pepstatin-aminohexyl agarose. (---), absorbance at 280 nm; Renin activity was determined before (●) and after (O) trypsin activation, and in the presence of anti-hog renal renin antibody after trypsin activation (Δ).

was completely inhibited by the addition of anti-hog renin antibody. These fractions were used as the inactive renin zymogen in the following studies. The second fractions (peak II-2, Fig. 2) eluted by 0.5 M Tris-buffer contained active renin, which was not activated by trypsin. The anti-hog rein antibody almost completely inhibited the enzyme activity. Thus, the completely inactive renin zymogen was separated from active renin.

Active renin fraction from the octyl-Sepharose column (Peak I-1, Fig. 1) was also purified further by the pepstatin-aminohexyl Sepharose column. Renin activity was eluted only by 0.5 M Tris-HCl, pH 7.5. The enzyme activity was inhibited by anti-hog renin antibody. Non-retained fractions did not exhibit renin activity even after the trypsin treatment, indicating the absence of inactive renin in these fractions.

Properties of inactive renin The molecular weight of inactive renin was estimated by gel filtration chromatography on a calibrated Sephadex G-100 column. The inactive renin was eluted in fractions corresponding to the molecular weight of 53,000 ± 2,000. Active renin generated from the inactive renin by trypsin treatment emerged at a position corresponding to 41,000 ± 1,000.

TABLE 1
ACTIVATION OF INACTIVE RENIN FROM HOG KIDNEY

ADDITION	ENZYME ACTIVITY ng angiotensin I formed/hr/ml	RELATIVE ACTIVITY (%)
None	0.05	-
Trypsin	133.0	100
Dithiothreitol	1.5	1.1
SDS	12.4	9.3
NaCl	10.6	8.0
Glycine-HCl	10.6	8.0

The inactive renin was treated with the following reagents: 50 mM dithiothreitol (pH 7.5, 37°C for 15 min), 3 M NaCl (pH 6.8, 4°C for 30 min), 0.005% SDS (pH 6.8, 37°C for 30 min) and 0.5 M glycine - HCl buffer (pH 3.3, 4°C for 18 hrs). These conditions known to dissociate the renin-inhibitor complex to a more active form (3, 4, 5, 6). None of these treatments caused significant activation as shown in Table 1.

DISCUSSION

The presence of activatable prorenin in plasma is well recognized. Recently we have shown that it is a totally inactive zymogen of renin with a molecular weight of 56,000 (15). In contrast, persistent failure in isolating totally inactive renin zymogen from the kidney was surprising in view of the fact that circulating renin is synthesized in this tissue.

Earlier observations by Boyd (4) and Leckie (3) and recent reports by Funakawa *et al* (5) and Murakami *et al* (6) indicated that high molecular weight renin (MW = 60,000) in the kidney may be a complex of active low molecular weight renin (MW = 40,000) and an inhibitory binding protein. This complex possesses a finite level of enzyme activity indicating the presence of a functional active site structure. It also undergoes reversible activation by 4 M NaCl (4), acidic pH (3) or sulfhydryl compounds (5). The high molecular

weight renin purified by Inagami and Murakami (9) has been found to be a complex of low molecular weight renin and other proteins. It does not have a capacity for an extensive activation.

In contrast, the inactive renin precursor, separated from hog kidney in the present studies is distinct from previously isolated high molecular weight, partially active renin in that it is totally inactive, that it undergoes marked activation by trypsin, and that it is not activated by procedures which causes dissociation of the renin-inhibitor complex. Its total lack of enzyme activity indicates that it is the zymogen of renin. Its high molecular weight (53,000) compared with that of active enzyme (40,000) also supported the notion that it is the zymogen. Thus, this is the first demonstration of inactive renin zymogen in the kidney. The presence of renin zymogen in hog kidney is compatible with the observation of inactive renin in hog plasma (16). The dissociable renin-inhibitor complex may be an intermediate in the proteolytic processing of the zymogen.

New affinity chromatographic procedures permitted rapid isolation and demonstration of the renin zymogen before its activation by renal proteases. In search of such a zymogen in the past, we have looked for an enzyme which binds to the pepstatin gel (9). Realization that inactive zymogen may not have the affinity to pepstatin has led us to the discovery of the zymogen in the non-retained fractions which are freed from active enzyme. No less important was the hydrophobic interaction chromatography on octyl-Sepharose which partially separated the active enzyme and inactive zymogen. Protease inhibitors and sulfhydryl blockers were used in the present isolation studies to prevent proteolytic activation of the zymogen. The latter is considered to promote the formation of a renin-inhibitor complex analogous to that formed by Na-tetrathionate as observed by Funakawa et al (5) and Murakami et al (6). However, the failure to observe reversible activation by dithiothreitol, 4 M NaCl, 0.005% SDS, or a pH 3.3 buffer of the present preparation, indicates that the zymogen isolated

here is not the product of such a complex formation since these conditions have been shown to dissociate the complex and caused marked activation (3, 4, 5, 6).

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