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ISOLATION OF COMPLETELY INACTIVE PLASMA PRORENIN AND ITS ACTIVATION BY KALLIKREINS

A POSSIBLE NEW LINK BETWEEN RENIN AND KALLIKREIN

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Summary

Existing views on prorenin are conflicting and its physiological activation mechanism is not clear. In an attempt to obtain clearcut views on the molecular properties of prorenin in human plasma, the renin zymogen (prorenin) was separated from active renin by two steps of affinity chromatography and it was demonstrated that prorenin is a completely inactive zymogen contrary to the existing information. Inactive prorenin has an apparent molecular weight of 56 000 contrary to 46 000—43 000 of partially active prorenin. Isolated and acid-treated human prorenin was shown to be activated by kallikreins from human urine and plasma. This activation was completely blocked by Trasylol. Hog pancreatic kallikrein also activated human prorenin. The kallikrein mediated activation of prorenin indicates the existence of a new link between the vasoconstricting renin-angiotensin system and the vasodilating kallikrein-kinin system.

Introduction

Renin (EC 3.4.99.19) is a highly specific peptidase which serves the single purpose of cleaving the decapeptide angiotensin I from the amino terminal portion of the α_2-globulin substrate angiotensinogen. As it catalyzes the rate

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limiting step in the renin angiotensin cascade, this enzyme plays a key role in blood pressure regulation.

Renin activity in various tissues has been found to increase upon exposure to acid pH [1—7], low temperature [8,9], high salt concentrations [4], and proteases such as trypsin and pepsin [2,4—6,10,11] and ion exchange chromatography [12]. This activation suggested the existence of renin zymogen or prorenin. Day and Luetscher have reported that a higher molecular weight (60 000) form of renin undergoes such activation [3,11]. However, the high molecular weight (60 000) form of renin purified partially or completely from kidneys failed to show such activation [13—15]. On the other hand partially purified prorenins from human plasma [16] and human amniotic fluid [17] were shown to belong to the low molecular weight forms (approx. 45 000). Thus there is a conflicting view on the molecular properties of prorenin.

Prorenin has often been called 'inactive renin'. However, all the activatable forms of renin studied to date have appreciable enzymatic activities. It has never been demonstrated that a completely inactive renin zymogen exists. Various conditions known to activate prorenin such as exposure to proteases, low pH and low temperature are not physiological. Yet, we know that the proportion of active renin to prorenin secreted into the blood stream varies depending on physiological demand for renin presumably through the activation of prorenin in the kidney [18,19]. Nothing is known about the physiological mechanism which mediates such activation. Thus, existing reports on the molecular and enzymological properties of prorenin are conflicting and fail to provide a clearcut view on prorenins.

Since the human plasma is the richest source of prorenin, we have initiated the present studies with the plasma prorenin as the first step to the elucidation of its molecular properties and of the mechanism of its activation. Completely inactive human prorenin was separated from active renin and its activation by kallikrein was demonstrated.

Materials and Methods

Separation of renin and prorenin: Two affinity chromatographic columns were used in sequence to separate inactive prorenin from active renin. Plasma (4 ml in 1 mM EDTA) from normotensive human subjects was applied to a 2.5 × 8 cm column of Cibacron Blue F3GA-agarose (20) (commercially available as Affigel Blue from Bio-Rad) in 0.02 M sodium phosphate buffer (pH 7.1), washed with 150 ml of the same buffer containing 0.2 M NaCl. Prorenin containing fractions were eluted by increasing NaCl concentration to 1.4 M. Prorenin containing fractions (Peak II) were equilibrated with 0.02 M sodium acetate buffer (pH 6.0) and applied to a pepstatin-aminohexyl-agarose column (2.0 × 6.0 cm) [21], washed with 60 ml of the same buffer followed by stepwise elution with 40 ml of the same buffer containing 1 M NaCl and finally with 0.5 M Tris-Cl buffer (pH 7.5). These isolation procedures were carried out at 4° C.

Activation of prorenin: to 0.1 ml of the fractionated plasma was added 10 μl of a freshly mixed solution containing 5 μg of trypsin (Worthington, TRL) and 0.5 mg of bovine serum albumin in 1 M Tris-Cl buffer (pH 7.5). After 10 min at

25° C trypsin was neutralized by addition of 50 μg of lima bean trypsin inhibitor. Activation of renin in unfractionated plasma was carried out with 0.1 mg of trypsin but without addition of bovine serum albumin.

Activation of prorenin by kallikreins was examined by allowing varying amounts of pure kallikrein to react with 0.1 ml of the fractionated plasma for 60 min in 0.1 ml of 0.1 M Tris-Cl buffer (pH 7.5) at 25° C. This tempeature was selected since reproducible activation was not obtained at 37° C between pH 7.5 and 8. Kallikreins used were pure human urinary kallikrein with a specific activity of 30 TAME units/mg, pure human plasma kallikrein prepared by activating pure prekallikrein with Hageman factor with a specific activity of 9.0 TAME units/A_{280} both supplied by Dr. J.J. Pisano, pure hog pancreatic kallikrein with a specific activity of 1180 TAME unit/mg supplied by Drs. E. Truscheit and G. Schmidt-Kastner and by Dr. H. Fritz, and another electrophoretically homogeneous hog pancreatic kallikrein preparation with a specific activity of 1300 TAME units/mg supplied by Seikagaku Kogyo of Tokyo, Japan. Pure human kallikrein supplied by Drs. E. Shaw and C. Kettner was also used in preliminary experiments. Pure human α-thrombin with a specific activity of 2600 NIH units/mg supplied by Drs. R.W. Downing and K. Mann was also tested for its ability to activate human prorenin. Pure clostripain was obtained from Worthington Biochemical Co. Pure Trasylol® was supplied by Bayer AG.

Prorenin was also activated by dialysis against 0.05 M glycine-Cl buffer (pH 3.0) containing 0.1 M NaCl for 20 h followed by a second dialysis against 0.1 M Tris-Cl (pH 7.5) containing 0.1 M NaCl for 20 h. Cryoactivation was examined by storing fractions at 0° C for a period of 5 days.

Renin activity was determined by radioimmunoassay of angiotensin I [22] generated during the incubation of the fractionated plasma (0.1 ml) with hog plasma substrate (2.8 ng angiotensin I equivalent) in 0.2 M Tris-Cl buffer (pH 7.5) at 37° C for an appropriate period of time.

Kallikrein inhibiting activity of fractionated plasma was determined by preincubating 15 μl fractions and 0.42 0.00042 TAME units of human urinary kallikrein in 50 μl of 0.1 M Tris-Cl buffer (pH 7.5) at 25° C for 30 min, then testing remaining kallikrein activity by the method of Margolius et al. using N^{α}-Tos-L-Arg-[^{14}C]OMe [23].

Molecular weight: The apparent molecular weight of prorenin was estimated by gel filtration on a column (85 × 2.5 cm) of Sephadex G-100 which had been calibrated with γ-globulin, bovine serum albumin, ovalbumin and soybean trypsin inhibitor. In addition, bovine serum albumin labeled with [1-^{14}C]-iodoacetic acid and ovalbumin labeled with [^{14}C]formaldehyde-borohydride [24] were run with samples as internal standards. It was confirmed that these radiolabeled standards coeluted with unlabeled bovine serum and ovalbumin, respectively, by separate experiments.

Results

Isolation of prorenin: Human plasma renin freshly obtained from normotensive subjects was separated into 2 renin fractions by affinity chromatography on a Cibacron Blue F3GA column (filled circles, Fig. 1, lower panel). Peak I, eluted without adhering to the column , accounted for a varying proportion (an

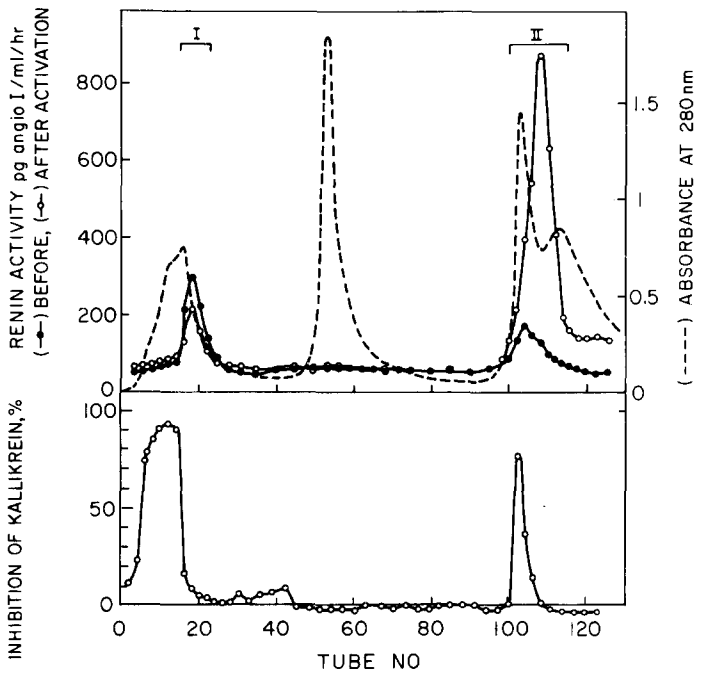

Fig. 1. Affinity chromatography of normal human plasma on an Affigel-Blue column. The column was eluted by 0.02 M sodium phosphate buffer (pH 7.1) with stepwise increases of NaCl concentration to 0.2 M and then to 1.4 M. The top panel shows protein concentration (------), renin activity before trypsin activation (•) and renin activity after the trypsin activation (○); the bottom panel shows the elution pattern of kallikrein inhibitory activity.

average of 60%) of the total renin activity in plasma. Peak II was eluted only at high salt concentration and accounted for a small portion of the total renin activity. Trypsin treatment of peak II fractions resulted in a marked increase in renin activity (open circles, Fig. 1 lower panel). On the other hand, peak I fractions did not undergo activation by the same treatment. Frequently the activity of this peak was slightly decreased by the trypsin treatment (open circles, Fig. 1 lower panel).

Plasma previously activated by trypsin did not produce the activatable prorenin fraction (peak II) upon chromatography on Cibacron Blue F3GA-agarose. The only renin fraction emerging from the column was active renin (peak I) which could not be activated further by additional trypsin treatment.

Various other treatments known to increase renin activity in plasma have been applied to active renin and prorenin. The enzyme activity of active renin (peak I, Fig. 1) was not increased (often slightly decreased) by dialysis at pH 3.0 followed by dialysis at pH 7.5. The acid treatment of prorenin (peak II, Fig. 1) resulted in extensive activation; however, the cold treatment at 0° C for 5—15 days [8,9] did not result in any activation. These observations suggested that peak I represented active renin and peak II prorenin. Wide variation was observed in the extent of the trypsin-mediated activation of prorenin ranging from 3- to 10-fold suggesting that this fraction was not homogeneous in its

renin to prorenin ratio. In order to obtain further separation of renin and prorenin, peak II fractions were pooled and applied to a column of pepstatin-aminohexyl-agarose and eluted by stepwise changes of buffers. Three fractions A, B and C were separated (Fig. 2). Peaks A and B did not exhibit renin activity (filled circles in lower panel) but the activity was generated by trypsin treatment. Renin activity could not be detected beyond the control level even with 5 times as much samples incubated for 12 h to generate angiotensin I. Peak C contained performed renin activity, which was not increased by the trypsin treatment. Thus, by the 2 step procedure, human plasma renin was separated into two major groups, one, completely inactive prorenin possessing a potential for activation and the other, already active renin whose activity is not increased by the trypsin treatment.

Molecular weight of inactive prorenin: The apparent molecular weights of prorenin both in peaks A and B were determined on the calibrated Sephadex G-G-100 column using the radiolabelled bovine serum albumin and ovalbumin as internal standards. It was found to be 56 000 ± 200. Active renin isolated from

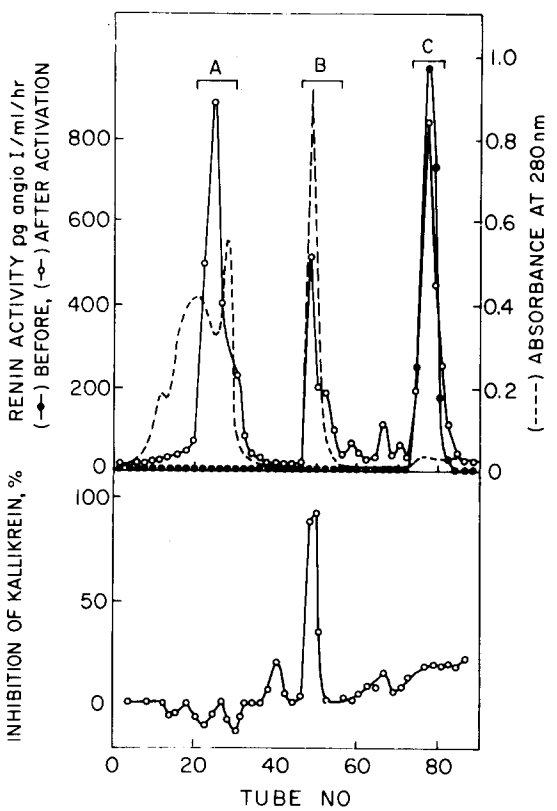

Fig. 2. Affinity chromatography of prorenin containing fractions obtianed from the Affigel-Blue column (Fig. 1 peak II) on a pepstatin-aminohexylagarose column. The elution buffer was changed to 0.02 M sodium acetate (pH 6.0) containing 1 M NaCl and then to 0.5 M Tris-Cl (pH 7.5). The top panel shows protein concentration (- - - - -), renin activity before trypsin activation (●) and renin activity after the trypsin activation (○); the bottom panel indicates the elution pattern of kallikrein inhibitory activity.

human kidney examined on the same column gave a molecular weight of 41 000.

Activation of prorenin: This completely inactive prorenin provided a convenient material to test various proteases for their ability to activate prorenin which may be functional under physiological conditions. Prior to the study of the prorenin activation by kallikrein, kallikrein inhibitory activity of each fraction separated by the affinity chromatography was estimated by using kallikrein. As shown in the lower panel of Fig. 1 both peak I and peak II contained appreciable kallikrein inhibitory activity. On the other hand prorenin containing peak A fractions obtained from the pepstatin-agarose column were practically free from the kallikrein inhibitors as assayed with Tos-Arg-OMe as substrate whereas peak B contained a large amount of the inhibitor. Therefore, the renin zymogen in peak A provided an ideal material for testing activation by kallikreins.

The renin zymogen (prorenin, approximately 0.15 ng) under peak A was activated by trypsin. But is was not activated by a large amount (5 μg) of urinary kallikrein nor by human plasma or hog pancreatic kallikreins as shown in the first column of Table I. Residual amounts of kallikrein inhibitors or inhibitors which inhibits protein substrates but not Tos-Arg-OMe in the sample

TABLE I

ACTIVATION OF PARTIALLY PURIFIED HUMAN PLASMA PRORENIN BY KALLIKREINS

	Renin activity (pg angiotensin I · ml^{-1} · h^{-1})	
	Prorenin	Prorenin after pH 3.3 treatment [a]
Control	109 [b]	479
Activation with		
Trypsin	1465 [c]	1235 [c]
Human urinary kallikrein		
1 μg/ml	109	1128
10	138	1822
25	144	1771
25 + Trasylol(R) [d]	—	489
25 + soybean trypsin inhibitor [e]	—	1384
Human plasma kallikrein		
1.2 μg/ml	109	407
6.1	101	803
13.3	—	1148
13.3 + Trasylol(R) [d]	—	443
6.1 + soybean trypsin inhibitor [e]	—	430
Hog pancreatic kallikrein B		
1 μg/ml	105	577
10	95	1420
25	—	1611
25 + Trasylol(R) [d]	—	468
25 + soybean trypsin inhibitor [e]	—	1153

[a] Dialyzed against 0.05 M glycine-Cl buffer (pH 3.3) containing 0.1 M NaCl overnight then adjusted to pH 8.0 with 2 M Tris-Cl buffer (pH 8.0).

[b] Due to small amount of renin contamination in hog renin substrate.

[c] Maximally attainable level with 1 μg trypsin in 5 min at 25°C.

[d] Trasylol(R) used at concentration of 35 μg/35 μl was preincubated with kallikrein for 20 min at room temperature.

[e] Soybean trypsin inhibitor (100 μg) was preincubated with kallikreins for 20 min at room temperature.

can prevent the kallikrein activation. In order to remove the inhibitors the prorenin fractions were dialyzed againt 0.05 M glycine-Cl buffer (pH 3.3) overnight at 4° C, since acidification is known to inactivate protease inhibitors [25]. This acid treatment produced some renin activity (1st line in the second column). However, the acid treated prorenin was activated by trypsin to the same level as the untreated material. As shown in the 2nd column the acid treated prorenin underwent a marked activation by human urinary kallikrein and by hog pancreatic kallikrein to the level comparable with that attained by trypsin activation. All hog kallikrein preparations were effective. Human plasma kallikrein also activated the acid treated prorenin markedly. The activation was completely suppressed by Trasylol®. Soybean trypsin inhibitor also suppressed the activation by plasma kallikrein, but activation mediated by human urinary kallikrein and hog pancreatic kallikrein was affected only slightly by this inhibitor.

Another plasma enzyme with trypsin-like specificity, human thrombin, did not exhibit any activating effect even at a concentration as high as 50 μg/100 μl.

Discussion

The present studies have clearly demonstrated the presence of inactive zymogen of renin for the first time. Furthermore, these studies have demonstrated the strong possibility that kallikrein or a kallikrein-like enzyme may participate in the activation of inactive prorenin. Studies on prorenin in the past were conducted mostly with unfractionated plasma or with partially fractionated samples in which active renin and prorenin were not completely separated. These studies left ambiguity in the interpretation of experimental results concerning the nature of prorenin. Boyd [16] and Shulkes et al. [17] made attempts to separate protenin from huma plasma and amniotic fluid by one step of ion exchange chromatography. These prorenin preparations, though often called inactive renin, exhibited considerable and variable enzyme activity like our peak II fractions. Thus, although the existence of inactive renin zymogen has been tacitly assumed, it has never been clearly demonstrated. Instead all the past observations indicated that prorenin was an active enzyme with a potential for further activation.

The complete separation of active renin from inactive prorenin by affinity chromatography in the present studies has shown that the active prorenin (peak II) is merely a mixture of active renin and its zymogen. Although it is probable that active prorenin isolated by Boyd [16] and Shulkes et al. [17] may be similar mixtures, the clear-cut difference in molecular weight between inactive prorenin obtained in the present studies (56 000) and active prorenins (45 000—43 000) [15,16] seems to suggest the latter preparations may be different substances. On the other hand, the molecular weight of renin zymogen found in the present study (56 000) agrees with the value (55 000) reported by Leckie et al. [26].

The molecular basis for the difference between active renin and prorenin in their behavior on the pepstatin column may reside in the imcomplete active site structure of prorenin as reflected by the diminished affinity of prorenin to pep-

statin which is an inhibitor of renin [27,28]. The molecular basis for the separation of prorenin into 2 inactive peaks (peaks A and B, Fig. 2) needs to be clarified by further studies.

Activation of prorenin by kallikrein is of great interest. Erodos and his collaborators have demonstrated that the vasoconstricting renin-angiotensin system and the vasodilating kinin-kallikrein system are linked by angiotensin I converting enzyme which is identical with kininase II [29]. The present studies provide evidence for the possibility that yet another link exists between these two systems. It is interesting to note that both of these links are directed to the increase of pressor activity.

The major function of kallikrein is limited to the proteolytic cleavage of kininogen. The present studies demonstrated that kallikreins react with yet another molecular species, prorenin, which does not seem to be related to kininogens.

The requirement of the acid treatment of prorenin suggests that residual amount of kallikrein inhibitors, which is not detected by the inhibition of Tos-Arg-OMe hydrolysis, may be still present in the prorenin fractions under peak A. A similar technique was shown to be useful to demonstrate kallikrein mediated increase of renin activity in unfractionated plasma by Sealey et al. in independent studies [30]. Whether the acidification has any direct effect on the prorenin molecule is not clear at this stage. The partial activation of prorenin induced by the acid treatment may be either due to plasma acid protease or due to autocatalysis by renin. The latter possibility is attractive since the chromatography of the pepstatin column may have removed other acid proteases in plasma. However, the removal of the acid proteases may not be complete. It is premature to speculate on the possibility of the autocatalysis by renin based on experiments using partially purified materials.

The amount of prorenin in each activation mixture was estimated as 0.15 ng from the maximum activity attainable by activation, the specific activity of pure human renin [31] and the molecular weights of prorenin and the active renin. Amounts of kallikrein and trypsin used as activator is 10 000—100 000 times as that of the renin zymogen. This may be most likely due to incomplete removal of kallikrein-trypsin inhibitors by the acid treatment. An excess amount of kallikrein or trypsin which may be needed to counteract or saturate the remaining endogenous inhibitors compared to very low concentration of prorenin makes kallikrein to prorenin ratio look very high.

Trasylol inhibited activation of prorenin by all three kallikreins whereas soybean trypsin inhibitor inhibited only the plasma kallikrein-mediated activation but not those catalyzed by glandular kallikreins. This pattern of inhibition agrees with the known pattern of kallikrein inhibition by these inhibitors and strongly indicates that the activation observed in the present studies is mediated by kallikreins and not by possible minor contaminant in the kallikrein preparations.

The presence of a large amount of kallikrein inhibitors in the plasma may not permit the activation of prorenin in the blood. However, it is known that renal kallikrein is present in the distal convoluting tubules including the macula densa in the juxtaglomerular apparatus [32,33]. It is possible that renal kallikrein may participate in the activation of prorenin in the kidney.

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