Studiees on the Activation and Molecular Weight of Inactive Renin in Human Plasma
KENICHI MORIMOTO, M.D., MASATO MATSUNAGA, M.D., AKIRA HARA, M.D., AND CHUICHI KAWAI, M.D.

SUMMARY The mechanism of activation of inactive renin was studied in normal human plasma. The molecular weight of active renin and those of inactive renin before and after activation were analyzed by sephadex gel filtration. Active renin of human plasma had a molecular weight of 48,000 ± 1000. Trypsin treatment and cold treatment activated inactive renin of a molecular weight of 54,000 ± 1000. The inactive renin apparently did not change its molecular weight after activation. "Cryoactivation" of inactive renin was possible only when whole plasma was used. When the whole plasma was fractionated by gel filtration, cryoactivation was not observed in any of the fractions. Cryoactivation requires certain plasma factor(s) contained in some fractions. Plasma kallikrein is likely to be a major factor required for the cryoactivation of inactive renin, whereas some other factors may also participate in this mechanism.

KEY WORDS • inactive renin • trypsin • cryoactivation • plasma kallikrein

THE PRESENCE of inactive renin in human plasma and its activation has been reported by many investigators. The occurrence of inactive renin was also shown in human amniotic fluid. An inactive form of renin "prorenin" in human plasma can be activated during storage at a low temperature (cryoactivation). Activation of inactive renin in plasma by acidification or by enzymes has also been shown. We have reported that certain serine enzyme(s) contained in plasma are responsible for the cryoactivation. In this study, we investigated further the mechanism of cryoactivation and molecular weight change of trypsin-activatable inactive renin.

Materials and Methods
Venous blood obtained from normal humans after 12 hours of fasting was added to 1 mg/ml disodium-ethylenediaminetetraacetate (EDTA-Na₂). Plasma was pooled and stored at −80°C until ready to use. A portion of the plasma was incubated with trypsin (Sigma, Type III) (0.5 mg/ml) at 25°C for 1 minute; then the reaction was stopped by soy bean trypsin inhibitor (Sigma) (0.5 mg/ml) (trypsin-treatment). Another portion was incubated at −5°C for 7 days (cold treatment).

For gel filtration, we used sephadex G-100 packed in a column 100 cm long with an inner diameter of 1.8 cm, and 50 mM pyrophosphate buffer, pH 7.5. The flow rate was 5 ml/hr obtaining every 2 ml of effluent. The column was calibrated by using blue dextran (Pharmacia), human γ-globulin (molecular weight, 156,000), bovine serum albumin (molecular weight, 67,000), and ovalbumin (molecular weight, 45,000). Molecular weights were estimated according to the method of Andrews. All the procedures were performed at 4°C, and molecular weights were estimated in three separate runs.

Untreated, cold-treated, and trypsin-treated plasma, each in an amount of 1 ml, were applied alternately to the column. Each fraction collected after gel filtration of untreated plasma was divided into three parts. One part was cold-treated, another part trypsin-treated. The remaining part received no treatment. To the untreated part of each fraction was added a fraction containing inactive renin (proved to be most activated by trypsin treatment) in an equivalent amount. One-half of the mixture was then cold treated.

Renin activity was assayed by a modification of Skinner's method. To 100 µl of each fraction, we added 10 mM of EDTA-Na₂, 5 µl of 5% (W/V) diisopropylfluorophosphate (DFP), 0.1 mg of cefalotin sodium, and 100 µl of exogenous excess sheep renin substrate. Then the mixture was incubated at 37°C for 10 hours. Generated angiotensin I was measured by...
radioimmunoassay (Haber et al.17) with a commercial kit from CEA-IRE-SORIN. We expressed the activity in terms of nanograms of angiotensin I liberated by 1 ml of a sample per hour (ng/ml/hr).

Plasma kallikrein activity was measured by a modification of the method of Morita et al.,18 employing a chromogenic substrate, carbobenzoxy-L-phenylalanil-L-arginine 4-methylcoumarinyl-7-amide (Z-Phe-Arg-MCA). Hydrolysis of this compound has been shown to be highly specific to the action of plasma kallikrein. Only a small percentage of this substrate is hydrolyzed by plasmin and factor Xa as compared with that by plasma kallikrein; α-thrombin and urokinase have no detectable action. A substrate-buffer solution of 2 ml of 50 mM tris-HCl buffer, pH 8.0, containing 100 mM NaCl and 0.1 mM Z-Phe-Arg-MCA, was preincubated for 10 minutes at 37°C, and then 100 µl of each fraction was added to it and mixed immediately. The reaction was carried out at 37°C for 60 minutes and terminated by adding 1 ml of 30% acetic acid. The activity of plasma kallikrein was expressed in terms of picomoles of 7-amino-4-methylcoumarin (AMC) liberated by 1 ml of a sample per hour (pm/ml/hr).

Results

The molecular weight of untreated plasma was estimated as 48,000 (SEM 1000; n = 3). After trypsin treatment of every fraction of untreated plasma, the peak of the increased renin activity was observed in the fraction corresponding to the molecular weight of 54,000 (SEM 1000; n = 3) (fig. 1). The peak of the increased renin activity of previously trypsin-treated plasma was also observed in the fraction corresponding to the molecular weight of 54,000 (SEM 1000; n = 3) (fig. 2). For the confirmation of the molecular weights before and after the trypsin activation of inactive renin, the inactive renin fraction was concentrated tenfold (Diafilter G 10 T), trypsin-treated, then applied to a sephadex G-100 packed column. One minute of trypsin treatment was enough for full activation of inactive renin. Further prolongation of the treatment led to a decline in renin activity. By the rerun of trypsin-treated inactive renin fraction, two peaks of renin activity were found. The major peak was found at the fraction corresponding to the molecular weight of about 54,000 and a minor peak was found at the fraction corresponding to the molecular weight of about 48,000 (fig. 3).

Cold treatment of whole plasma activated approximately 50% of the trypsin-activatable inactive renin. The peak of the increased renin activity of the previously cold-treated plasma was found at the fraction corresponding to the molecular weight of 54,000 (SEM 1000; n = 3) (fig. 4). After cold treatment of every fraction of untreated plasma, renin activity of each fraction remained almost unchanged, i.e., cryoactivation was not observed in any of the fractions by its own (fig. 5). After fractionation of untreated plasma, the fraction that contained inactive renin was added to each of the remaining fractions in an
After collecting the inactive renin fractions (No 52 in fig. 1), it was concentrated tenfold, trypsin-treated, then applied to a sephadex G-100 packed column (1.8 X 100 cm) and eluted with 50 mM pyrophosphate buffer, pH 7.5. Renin was assayed for each fraction (open circle). The renin activity was expressed in terms of nanograms of angiotensin I per ml per hour. Peaks of the absorbance at 280 nm of the molecular weight standards are indicated by arrows: human y-globulin (156,000), bovine serum albumin (BSA, 67,000), and ovalbumin (OVA, 45,000).

**FIGURE 5.** Cold treatment after gel filtration of untreated plasma. Human plasma (1 ml) was applied to a sephadex G-100 packed column (1.8 X 100 cm), eluted with 50 mM pyrophosphate buffer, pH 7.5. A portion of each fraction was cold-treated. Another portion received no treatment. Renin was assayed for untreated fractions (black circle) and for cold-treated fractions (open circle). The renin activity was expressed in terms of nanograms of angiotensin I per ml per hour. Peaks of the absorbance at 280 nm of the molecular weight standards are indicated by arrows: human y-globulin (156,000), bovine serum albumin (BSA, 67,000), and ovalbumin (OVA, 45,000).

Renin activity of each mixture before and after cold treatment and plasma kallikrein activity of each fraction before cold treatment are shown in figure 6. The main occurrence of cryoactivation was observed at the fractions corresponding to the molecular weights of more than 70,000, with minor peaks at fractions of smaller molecular weights. Plasma kallikrein activity was found at the fractions with the molecular weights of more than 70,000. This activity was not inhibited by 5 µg/ml lima bean trypsin inhibitor (Sigma), whereas it was completely inhibited in the presence of 5 µg/ml soy bean trypsin inhibitor. This coincides with the characteristics of plasma kallikrein (Bagdasarian et al.). Involvement of trypsin and plasmin in this reaction is excluded (Ohishi and Katori), because these enzymes are susceptible to both of the inhibitors. Involvement of trypsin and factor Xa is also excluded because of their smaller molecules. Furthermore, activation of factor X and subsequent pathway of activation of coagulation factors, requiring calcium ions, are considered to have been protected by the presence of EDTA. Thus, the hydrolysis observed here can be attributed mostly to the action of plasma kallikrein.

To a 200 µl portion of the mixture of plasma kallikrein-containing fraction and inactive renin-containing fraction was added 5 µl of 5% (W/V) DFP, and the mixture was then cold treated for 7 days. The other portion of the mixture was incubated at 25°C.
for 10, 20, 30 and 60 minutes. Plasma kallikrein activity in the mixture was inhibited almost completely by adding DFP, and renin activity was not increased. Plasma kallikrein activity was nearly unchanged when the mixture was cold-treated without DFP. When incubated at 25°C for 1 hour, plasma kallikrein activity was nearly lost, and no significant increase of renin activity was observed (fig. 7).

Discussion

The activation mechanism of inactive renin is still controversial. Cold treatment, trypsin treatment, and acid treatment has been used for activation of inactive renin. In this study, we employed trypsin treatment and cold treatment.

The molecular weight of active renin in normal human plasma that we obtained was 48,000 ± 1000, which is somewhat greater than that reported by others (molecular weight 46,000, Shulkes et al., molecular weight 43,000 ± 1000, Day and Luetscher, molecular weight 41,000 ± 1000, Boyd). This discrepancy in the molecular weight estimated by gel filtration may be derived from the use of crude material for analysis of molecular weight of plasma renin.

When the untreated plasma was fractionated, and then treated with trypsin, the most activatable fraction, i.e., the fraction assumed to contain inactive renin, had a molecular weight of 54,000 (SEM 1000; n = 3). When previously trypsin-treated plasma was fractionated, the peak of renin activity was also found at the molecular weight of 54,000 (SEM 1000; n = 3). Those results suggested that inactive renin did not change its molecular weight by trypsin activation. It was confirmed by an experiment in which the fraction of untreated plasma containing inactive renin was concentrated, trypsin-treated and then renz on sephadex G-100. The major peak was found at the fraction corresponding to the molecular weight of 54,000. The small peak at the molecular weight of 48,000 may be due to the active renin that was contained in the original inactive renin fraction, although the possibility that it represents one of the activated forms of inactive renin cannot be excluded.

The molecular weight of inactive renin varies depending on investigators. Hsueh et al. showed two forms of inactive renin (molecular weight of 60,000 and that of 40,000 in human plasma of salt-loaded subjects) activatable by exposure to pH 3.3. Boyd reported inactive renin with a molecular weight of 43,000 in normal human plasma, which can be converted to an active form by acidification to pH 3.3. Shulkes et al. reported acid-activatable inactive renin with a molecular weight of 46,000 in human plasma. Leckie et al. showed that inactive renin with a molecular weight of 55,000 in the plasma of patients with renal artery stenosis or pregnant women was converted to an active form with a molecular weight of 37,000–38,000 by acidification. All of these reports were based on acid activation.

In this study, we employed trypsin activation for the detection of inactive renin. Our results showed the presence of inactive renin in a molecular weight of 54,000 ± 1000 in normal human plasma, and trypsin activated it without apparent change in molecular weight. The reason for the difference in molecular weight of inactive renin and its product may be due to a difference in the mechanism between acid activation and trypsin activation, and probably to the presence of multiple types of inactive renin. Weinberger et al. showed that a renin in anephric human plasma had the same molecular weight of 59,000–61,000 before and after acidification. No change in molecular weight before and after activation of big renin (molecular weight 63,000 ± 2000) in plasma of patients with Wilms' tumor was reported by Day and Luetscher. In our present study, the molecular weight of trypsin-activated inactive renin (54,000 ± 1000) was greater than that of active renin (48,000 ± 1000). Therefore, it is possible that big active renin found in human plasma may be an activated form of the inactive renin.

We already reported an involvement of serine enzyme(s) in the activation of inactive renin during cold treatment. The present results confirmed that some plasma factors are necessary for cryoactivation of inactive renin. Although cold treatment activated inac-
tive renin in the whole plasma, cryoactivation did not occur in the fractions obtained by gel filtration of the same plasma. When the fraction containing inactive renin was mixed with each of the other fractions and then cold-treated, cryoactivation was observed with many fractions. The highest elevation of renin activity was found with the fractions corresponding to more than 70,000 of the molecular weight in which plasma kallikrein activity was present. When DFP, a serine protease inhibitor, was added to the mixture of plasma kallikrein and inactive renin fractions before the cold treatment, plasma kallikrein activity was nearly inhibited and cryoactivation did not occur. Why the cold treatment is needed and why there is little activation of inactive renin when the mixture was incubated at 25°C for 1 hour can be explained by the loss of plasma kallikrein activity during incubation at 25°C, whereas it remained almost intact during cold treatment. These results suggest that plasma kallikrein, one of the serine proteases, may be the major factor required for the cryoactivation of inactive renin. However, Osmond et al. and Millar et al. showed cryoactivation in Fletcher factor (prekallikrein) deficient plasma. Occurrence of cryoactivation with some fractions in which no plasma kallikrein activity was detected in the present study also indicated an involvement of some other enzymes.

Plasma kallikrein circulates as an inactive form, prekallikrein, probably in a complex with high molecular weight kininogen (Mandle et al.). In the case of whole plasma, it has been known that a low temperature activates prekallikrein (Gjønnes and Stormore; Derkx et al.). We observed parallel increases in plasma kallikrein and renin activities during the cold treatment of whole plasma (unpublished data). Presence of active kallikrein in the fractions obtained by gel filtration can be explained by partial activation of prekallikrein during the experimental procedures. Contact with active surface during the gel filtration can activate Hageman factor and consequently prekallikrein (Ogston and Bennett). Low temperature may also contribute to the activation. More than one peak of plasma kallikrein activity may be due to the presence of kallikrein molecules in forms combined with and free from high molecular weight kininogen, although an involvement of other unidentified enzymes in the MCA-hydrolyzing activity cannot be excluded. Because sephadex G-100 gel filtration is less able to discriminate protein molecules close to the exclusion limit of molecular weight 150,000, further identification is needed.

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