Biochemical Similarity of Expressed Human Prorenin and Native Inactive Renin

Willa A. Hsueh, Yung Shun Do, Tatsuo Shinagawa, Helen Tam, Phyllis A. Ponte, John D. Baxter, John Shine, and Lawrence C. Fritz

SUMMARY  Prorenin is secreted by mammalian cells transfected with a human preprorenin expression construct. The purpose of this investigation was to compare the physicochemical properties of expressed prorenin in culture medium with the known characteristics of human inactive renin, which accounts for nearly half the renin in plasma and kidney. We found that expressed human prorenin strongly resembles human renal and plasma inactive renin. The expressed prorenin was inactive and could be equally activated by acid (dialysis to pH 3.3) or trypsin. Acid activation was completely reversible; reexposure to acid could reactivate the expressed inactive renin. Exposure to cold (—5°C for 3 days) could also activate expressed renin. The Michaelis-Menten constant of acid-activated expressed renin with sheep substrate was 0.29 μM, and the pH optimum was 7.8. Expressed inactive renin bound to a cibacron-blue affinity column and could be eluted with 0.5M NaCl. All the above characteristics resemble those of human renal and plasma inactive renin. In addition, the molecular weight of expressed prorenin and human chorionic renin was 47,000, as determined by sodium dodecyl sulfate—polyacrylamide gel electrophoresis, and 46,000, as measured by high-performance liquid chromatography. These data, taken together with the published observation that native human inactive renin cross-reacts with antibodies generated against amino acid sequences in the prosegment of renin, provide strong support for the hypothesis that human inactive renin is prorenin.

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KEY WORDS  • expressed human prorenin • inactive renin

In humans more than half the renin in the circulation exists as an inactive form.1,2 Even higher levels of circulating inactive renin are found in some patients who have defects in active renin secretion, such as in the syndrome of hyporeninemic hypoaldosteronism.3,4 Indeed, it has been suggested that the syndrome may result from an inability to convert inactive renin to active renin. To understand such defects in renin secretion, it is important to identify the physiological role of inactive renin and to understand the relationships between the active and inactive forms of the enzyme.

From the Section of Endocrinology, Los Angeles County/University of Southern California Medical Center, Los Angeles (W.A. Hsueh, Y.S. Do, T. Shinagawa, H. Tam); California Biotechnology, Inc., Mountain View (P.A. Ponte, J. Shine, L.C. Fritz); and the Metabolic Research Unit, University of California, San Francisco (J.D. Baxter), California.

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Address for reprints: Willa Hsueh, M.D., LAC/USC Medical Center, 2025 Zonal Avenue, Unit I 18-632 Los Angeles, CA 90033.

The question of whether or not human inactive renin is a biosynthetic precursor of renin (i.e., prorenin) has been controversial. Several studies provide indirect evidence to support the prorenin nature of inactive renin. First, it has been observed that renin-secreting tumors synthesize and secrete large quantities of inactive renin,5-7 like polypeptide hormone-secreting tumors, which produce large quantities of prohormone. Second, absolute levels of inactive renin have been found to change with perturbation of the renin system; for example, with marked, acute stimulation of renin, such as that which occurs with inhibition of converting enzyme, there is a reciprocal drop in inactive renin while active renin rises.8 Third, antibodies developed against pure human renal renin cross-react with inactive renin.9,10 Fourth, pulse-labeling studies in mouse submandibular gland, which makes large quantities of renin, indicate that renin is synthesized in two forms — preprorenin and prorenin, both of which are inactive.11-13 Fifth, peptide mapping suggests that human renal inactive renin is a renin zymogen.14 Finally, antibodies generated against synthetic peptides derived from the prosegment of human prorenin react with human inactive renin.15-17

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This evidence, however, cannot exclude the alternative hypothesis that inactive renin represents active renin bound to an inhibitor protein. Indeed, these "latent" forms of renin, as labeled by Inagaki and Inagami, have been extensively described in animal plasma and tissue.

Recently, human kidney renin complementary DNA (cDNA) and the human renin gene have been cloned and sequenced. This has allowed deduction of the amino acid sequence of preprorenin. Using cloned cDNA, Fritz et al. have constructed a eukaryotic expression plasmid that directs the synthesis of human preprorenin.18 This plasmid was shown to secrete an active renin into the culture medium, which could subsequently be activated with trypsin. Using this material, we have been able to make a direct comparison between prorenin and inactive renin, and we report herein that they demonstrate a striking biochemical similarity.

**Methods**

Active renin was measured by radioimmunoassay of angiotensin I after incubation of samples with sheep angiotensinogen at 37°C, pH 7.5, in the presence of angiotensinase inhibitors. Total renin was measured by two different methods: one using low pH, and the other using trypsin. Briefly, the supernatant was dialyzed against 0.05 M glycine buffer, pH 3.3, at 4°C for 18 hours, quickly neutralized, and incubated with sheep angiotensinogen as above for 0, 10, 20, and 30 minutes. The slope of angiotensin I concentration versus time was determined by linear regression analysis (r > 0.96 was considered acceptable). Trypsin (Sigma Chemical, St. Louis, MO, USA) was used according to a modification of the method of Atlas et al. in a concentration of 500 µg/ml at 0°C in the presence of 200 mM benzamidine.

Fritz et al. cloned a cDNA sequence coding for human prorenin from a human kidney cDNA library. The cDNA was expressed in CHO cells in culture with a mammalian cell expression system. This resulted in the secretion of prorenin into the culture medium, which consisted of Dulbecco's minimal essential medium 21/Coon's F12 (1:1), 10% fetal calf serum, and antibiotics. Characterization of prorenin secreted into the culture medium included: 1) comparison of trypsin, acid, and cold activation; 2) confirmation of the presence of reversible acid activation; 3) determination of the pH optimum of activity with sheep angiotensinogen as substrate; 4) determination of the Michaelis-Menten constant, K_m, with sheep angiotensinogen at pH 7.5; and 5) a test of the ability to bind to cibacron-blue. These methods have previously been described in detail.24 Cryoactivation was performed by incubating the culture supernatant at −5°C for 4 days. To reverse acid activation, culture supernatant that had been dialyzed to a low pH was incubated at 37°C, pH 7.5, for 4 hours. Reactivation occurred with redialysis against a low pH. Binding to cibacron blue was tested by applying 1.5 ml of supernatant to a 0.5 × 6-cm cibacron blue affinity column. After application of the sample, the column was washed with 0.025 M phosphate buffer, pH 7.0, and eluted with a gradient of 0 to 1.0 M NaCl.

In addition, the molecular weight of the expressed prorenin was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), followed by Western blotting and immunoanalysis, and gel filtration high-performance liquid chromatography (HPLC). SDS slab gels containing 12.5% acrylamide were made according to the method of Laemmli, and stained with Coomassie blue. Protein standards (BioRad, Concord, CA, USA) included phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). For Western blotting, proteins were separated on SDS–polyacrylamide gels and blotted onto nitrocellulose paper, using the sandwich technique of Burnette. The nitrocellulose paper was incubated at 25°C for 1 hour with rabbit polyclonal antibody generated against the last 12 amino acids of the prosegment of prorenin; this antiserum is known to cross-react with human inactive renin. Immunoglobulin bound to the nitrocellulose was visualized with a horseradish peroxidase system (BioRad). CHO supernatant (50 ml) was applied directly to SDS. Native inactive renin was extracted from human chorionic homogenate.

Gel filtration was performed on a BioRad HPLC system with a TSK 3000 (Aittech, Deerfield, IL, USA) column. One hundred microliters of either CHO supernatant or amniotic fluid was applied to the column and eluted with 50 mM NaHPO_4_ and 0.5M NaCl, pH 6.5, containing 2% C_2H_6OS. This concentration of C_2H_6OS had no effect on the renin assay. The flow rate was 0.5 ml/min. Protein standards (BioRad) were bovine thyroglobulin (670,000), immunoglobulin (lg) G (158,000), ovalbumin (45,000), myoglobin (17,000), and B_2 (1350). Eluates were activated by acid dialysis and assayed for total renin.

**Results**

**Effect of Acid and Trypsin**

The active renin concentration in the untreated culture supernatant was 370 ng/ml/hr. Treatment with acid resulted in a total renin concentration of 25.425 ng/ml/hr (Figure 1), and treatment with trypsin yielded a similar value: 22,770 ng/ml/hr. After overnight dialysis of the supernatant against pH 3.3 buffer, neutralization, and incubation at 37°C for 3 hours, the renin concentration decreased to 6% of the acid-activated level. Redialysis of the sample against the pH 3.3 buffer completely reactivated the sample. Incubation at −5°C for 4 days increased the concentration to 18,120 ng/ml/hr, or 71% of the level determined by acid. Details of reversible acid activation are shown in Figure 2. Reversible acid activation and reactivation occurred in the presence or absence of aprotinin, 200
Effect of acid, cold, and trypsin on expressed protein. Chinese hamster ovary supernatant was treated as indicated beneath each bar (see text for details).

Reversal at 37°C occurred within 1 hour. Reactivation by low pH at 4°C required 5 to 8 hours.

Kinetic Studies
Prorenin from the culture medium was activated by dialysis against pH 3.3 buffer and human renal kallikrein, as previously described. The optimum pH for enzymatic activity with sheep angiotensinogen was 7.8. The K_m with sheep substrate was determined to be 0.29 μM.

Cibacron Blue Binding
When the supernatant was applied to cibacron blue, active renin did not bind, whereas prorenin did (Figure 3). The bound prorenin eluted at a concentration of 0.5 M NaCl, as demonstrated by acid activation of the eluates. Recovery of prorenin from the column was 71%.

Determination of Molecular Weight
SDS-PAGE and immunoblot analysis are shown in Figure 4. Prosegment antibody cross-reacted with both expressed prorenin and human chorionic renin. The molecular weight for both renins, determined by a plot of log of molecular weight versus retardation factor was 47,000. HPLC yielded a similar value of molecular weight — 46,000 — for expressed prorenin and inactive renin in amniotic fluid (Figure 5), as did SDS-PAGE.

Discussion
Expressed human prorenin and native human inactive renin are similar in their activation characteristics, molecular weight, kinetic activity, and binding to a cibacron blue affinity column (Table 1). These data, taken together with the observation that native human inactive renin cross-reacts with antibodies generated against amino acid sequences in the prosegment of prorenin, provide strong support for the hypothesis that human inactive renin is prorenin.

Reversible acid activation is a hallmark of human inactive renin. It occurs in inactive renin extracted from plasma, kidney, amniotic fluid, and chorion. The process appears to be unimolecular and is not dependent on other proteases in the system. Acid exposure induces a conformational change in inactive renin such that the active site is exposed. The critical pH at 4°C is 3.3; incomplete activation occurs at a higher pH. With neutralization and an increase in the temperature to 37°C, a time-dependent loss of enzyme activity is observed.
activity occurs, consistent with a refolding of the molecule to its original conformation. With reexposure to pH 3.3, the enzyme becomes reactivated. In plasma and kidney, the presence of aprotinin is necessary to demonstrate reversible acid activation, since kallikrein in plasma and kidney irreversibly activates renin. The reversible acid activation observed with expressed prorenin was independent of aprotinin, reflecting the relative absence of kallikrein-like activating proteases in the supernatant. As with inactive renin, trypsin treatment and acid dialysis were roughly equivalent in their ability to activate prorenin. Cold incubation activated prorenin to 71% of the concentration with acid dialysis, whereas cryoactivation of plasma inactive renin yields about 30% activation. This discrepancy could reflect differences in inactive renin preparations and culture supernatants. Enzymes in fetal calf serum may be responsible for the increased cryoactivation of expressed prorenin. The mechanism of cryoactivation remains unknown.

Binding to a cibacron blue affinity column is also a unique feature of human inactive renin. The prosegment is required, since active renin does not bind. This technique is thus useful in separating active from inactive renin. The nature of the binding is unknown. Expressed human prorenin binds readily to cibacron blue and is eluted at 0.5 M NaCl (when a gradient of NaCl is employed), which is similar to the elution pattern of human inactive renin.

The molecular weight of human inactive renin has been reported to range from 40,000 to 60,000, depending on the fractionation technique used and the method of subsequent activation of renin. Use of Sephadex
G100 generally yields values of 48,000 to 55,000, whereas use of other molecular sieving gels and SDS–PAGE yields lower values of molecular weight. McIntyre et al. reported that the molecular weight of human renal inactive renin was 48,000, as determined by SDS–PAGE, and 51,000, as determined by gel filtration on Sephadex G100 with trypsin treatment of the eluates. Human prorenin expressed in CHO cells is a doublet of 49,000 and 50,000, as demonstrated by immunoprecipitation of prorenin from biosynthetically radiolabeled supernatants followed by SDS gel electrophoresis. Our estimation of molecular weight by SDS-PAGE yields lower values of molecular weight. Whereas use of other molecular sieving gels and HPLC. Expressed human prorenin and native inactive renin have a molecular weight of 47,000 by SDS-PAGE and 46,000 by HPLC.

The $K_m$ and pH optimum for expressed human prorenin with sheep substrate are also similar to those reported for human renal and plasma inactive renin and for human renal active renin. Despite the fact that renin is an acid protease, the pH optimum for expressed human prorenin with sheep angiotensinogen is near neutrality. The $K_m$ of human renin with sheep substrate is one third the $K_m$ of human renin with human substrate.

The biosynthetic processing of renin has been studied in mouse submaxillary gland, one of the richest known sources of renin. Cell-free translation and pulse labeling indicate that the primary translation product of renin messenger RNA is preprorenin. During translocation into the rough endoplasmic reticulum, preprorenin is cleaved to prorenin, which is then converted to renin in the Golgi complex. Studies of human renin-secreting tumor cells demonstrate that tumoral renin is synthesized as a 55,000-molecular-weight inactive form, which is then converted to a 44,000-molecular-weight active renin. However, when cells from this tumor were cultured, the 55,000 form was exclusively released into the medium, and no conversion to the smaller form occurred. Galen et al. postulated the existence of two pathways for the secretion of human renin: in one prorenin is packaged into secretory granules where it is processed into active renin, and in the other prorenin is not stored or processed but is released directly by the cell. These pathways are analogous to the “regulated” and “constitutive” pathway described in pituitary tumor cells. The latter pathway, proforms are not stored but released by the cell. If the constitutive pathway is normally operative in human juxtaglomerular cells, it may explain the large quantities of inactive prorenin in human plasma.

Cloning and sequencing of the human renin gene indicate that the prosegment of human renin consists of a 46-amino acid peptide. In an attempt to determine whether inactive renin is prorenin, several investigators developed antibodies to fragments of the prosegment and demonstrated that they bind human inactive renin. Bouhnik et al. synthesized a 13-amino acid segment of this peptide (Glu 28 → Trp 40) and generated polyclonal antibodies to the fragment. When the antibodies were purified and coupled to an affinity gel, they appeared to bind inactive renin from human kidney and plasma. However, elution with 6 M urea destroyed the activity of the bound renin, which could then be demonstrated only by direct radioimmunoassay using an enzyme-linked immunosorbent assay. Presumably, inactive renin was measured in the eluates containing bound protein. Atlas and colleagues developed a polyclonal antibody to the last 12 amino acids of the prosegment (Arg 35 → Arg 46). Antibody binding to plasma and renal inactive renin could be demonstrated by using protein A to precipitate the antibody and by finding no activatable renin activity in the supernatant. Kim et al. developed an antibody to a 15-residue peptide in the prosegment (Asp 32 → Lys 45) and demonstrated that it cross-reacted with both human plasma inactive renin (binding to an immunoaffinity column) and human preprorenin obtained from cell-free translation of poly(A) + RNA, isolated from ischemic human kidney in a rabbit reticulocyte lysate system.

The present studies strongly support the findings of these previous investigations. Ultimate identification

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<th>TABLE 1. Comparison of Physicochemical Properties of Expressed Human Prorenin and Native Inactive Renin</th>
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<td><strong>Expressed prorenin</strong></td>
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$K_m$ = Michaelis-Menten constant; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HPLC = high-performance liquid chromatography.

*Taken from Hsueh et al.24
EXPRESSED HUMAN PRORENNIN/Hsueh et al.

of human inactive renin lies in its complete purification and amino acid sequencing. However, the lability of inactive renin and its susceptibility to activation render purification in large quantities a difficult task. Nevertheless, the identification of human inactive renin as prorenin is a major step in determining the physiological importance of human inactive renin and in defining defects in renin secretion in humans.

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