Similarity between physicochemical properties of recombinant rat prorenin and native inactive renin

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Rat prorenin was synthesized by Chinese-hamster ovary cells transfected with an expression vector containing rat preprorenin cDNA sequences, then purified by concanavalin A–Sephadex chromatography and h.p.l.c. on G3000SW. The molecular mass of purified prorenin was 46000 Da, as determined by h.p.l.c. on G3000SW. Immunoblot analysis indicated that recombinant prorenin cross-reacted with anti-(mature renin) antibody and two kinds of antibodies recognizing the *N*-terminus and *C*-terminus of the prosegment of rat prorenin. Recombinant prorenin was bound to a Cibacron Blue–Sephadex column and eluted with 1.4 M-NaCl, but was not retained by an octapeptide renin inhibitor (H-77)–Sephadex column. Trypsin activation of prorenin increased the renin activity 110-fold, caused binding to an H-77–Sephadex column and nullified the reactivity to the above two kinds of anti-prosegment antibodies, findings indicating that the activation of prorenin with trypsin is due to the cleavage of the prosegment. Rat plasma inactive renin, partially purified by h.p.l.c. on G3000SW, had much the same physicochemical characteristics as the recombinant prorenin. These results provide evidence that rat plasma inactive renin is prorenin. Recombinant prorenin is a useful material for examining the physiological role of circulating prorenin.

INTRODUCTION

Renin (EC 3.4.23.15), an aspartic proteinase mainly synthesized by the kidney and released into the blood, is a key enzyme in the renin–angiotensin–aldosterone system and plays a critical role in the regulation of blood pressure and electrolyte balance [1].

In humans and various animals including rats, more than half of the renin in the plasma exists in an inactive form, and it can be readily activated *in vitro* by various kinds of proteinase, acidification or cold exposure [2,3]. There is now evidence that most of the human plasma inactive renin, if not all, is prorenin, the biosynthetic precursor of renin [4–7].

We injected into monkeys recombinant human prorenin, synthesized by Chinese-hamster ovary cells, the objective being to search for possible activation of prorenin *in vivo*. We found that the activation of prorenin does not occur in the blood but rather in the liver and kidney [8]. However, further study on the mechanism of activation of prorenin *in vivo* has been hampered by the lack of a suitable animal model.

In the present study, recombinant rat prorenin was purified from the culture medium of Chinese-hamster ovary cells transfected with the rat renin gene [9]. We obtained evidence that recombinant rat prorenin has physicochemical properties similar to those of the native rat plasma inactive renin.

MATERIALS AND METHODS

Chemicals

BSA, methyl α -mannoside, trypsin from bovine pancreas [type III; 10200 BAEE (*N*^α-benzoyl-L-arginine ethyl ester) units/mg] and soya-bean trypsin inhibitor (type I-S) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cibacron Blue–Sephadex (100–200 mesh) and nitrocellulose membranes were from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

Concanavalin A–Sephadex 4B and Sephadex G-25 were from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). G3000SW h.p.l.c. columns were from Tosoh Co. (Yamaguchi, Japan). The octapeptide renin inhibitor H-77 was purchased from the Peptide Institute (Osaka, Japan). Centricon-10 was from Amicon (Danver, MA, U.S.A.). Protein A–cellulofine was from Seikagaku Kogyo (Tokyo, Japan). Vecstatin ABC kit was from Vector (Burlingame, CA, U.S.A.).

Plasmid construction and DNA transfection

A 1.4 kb *Xba*I–*Nsp*(7524)I cDNA fragment covering the entire coding sequence of rat preprorenin [10] was blunt-ended with T4 DNA polymerase, ligated with *Bam*HI linkers and digested with *Bam*HI. An expression plasmid of rat preprorenin, pSVRRn1 (Fig. 1), was constructed by exchanging the cDNA fragment of human preprorenin in pSVDPRnPA33 [11] with the *Bam*HI–*Bam*HI rat preprorenin cDNA fragment. pSVRRn1 was transfected into Chinese-hamster ovary cells (DXB-11 strain), which are defective in dihydrofolate reductase, by calcium phosphate co-precipitation. Stable transfectants were selected in a thymidine-free medium [Dulbecco's modified Eagle's medium supplemented with 11.5 mg of proline/l and 10% (v/v) dialysed fetal-calf serum]. Clonal cell lines were screened for secretion of prorenin into the culture media. The highest-prorenin-producing cell line was cultured in the serum-free medium S-Clone SF-O (Sanko Pure Chemical, Tokyo, Japan) for 3 days, and the culture medium was subjected to purification of rat prorenin.

Purification of recombinant rat prorenin

The serum-free culture medium (100 ml) was applied to a concanavalin A–Sephadex column (0.76 cm × 3.3 cm) equilibrated with phosphate-buffered saline (PBS), and eluted with PBS containing 0.2 M-methyl α -mannoside at 4 °C, as reported previously [12]. The fractions containing prorenin were pooled and concentrated by centrifugation in Centricon-10.

Abbreviations used: PBS, phosphate-buffered saline (0.15 M-NaCl/20 mM-sodium phosphate buffer, pH 7.4); AI, angiotensin I.

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Fig. 1. Diagram of the expression plasmid of rat prorenin

Psv, SV40 early promoter, Rat renin, rat preprorenin cDNA; poly(A), the polyadenylation signal from bovine growth hormone gene; Amp^R , β -lactamase gene from pBR322; DHFR, mouse dihydrofolate reductase gene.

The fraction of prorenin, eluted from a concanavalin A-Sepharose column, was chromatographed on a G3000SW column (0.75 cm $\times$ 60 cm), by using our previous method [12]. Fraction (500 μ l) were collected into plastic tubes in which BSA at a final concentration of 0.02% (w/v) was included to stabilize the prorenin. The fractions containing prorenin were pooled and concentrated by centrifugation in Centricon-10, to give a preparation that was used as purified recombinant rat prorenin for the characterization.

Preparation of rat plasma inactive renin

Male Wistar rats (12 weeks old; Clea, Tokyo, Japan) were used. Blood was collected, after decapitation of the conscious rats, into plastic tubes containing EDTA (1 mg/ml). The plasma was obtained by centrifugation at 1500 *g* for 20 min at 4 °C. To purify the inactive renin partially, a plasma sample (500 μ l) was subjected to h.p.l.c. on a G3000SW column [12]. Fractions (500 μ l) were collected into plastic tubes containing BSA at a final concentration of 0.1%. The above chromatographic procedure was performed ten times and the fractions containing inactive renin were pooled; this served as the partially purified plasma inactive renin for the characterization.

Renin activity

Renin activity was determined as the rate of angiotensin I (AI) formation [13], which was measured by radioimmunoassay.

Activation of inactive renin with trypsin

Total renin (inactive renin plus active renin) in the sample was measured after activation of inactive renin by trypsin, under optimal conditions [14]. Inactive renin was calculated as the difference between total renin and active renin.

Preparation of anti-(prorenin prosegment) antibodies and anti-(mature renin) antibody

Two kinds of antiserum against the prosegment sequence of rat prorenin deduced from the rat renin cDNA [9] were produced in rabbits. One was raised against the N-terminal 15-amino acid-residue sequence (Pro N; LPTDTASFGRIKK) of the

prorenin prosegment and termed anti-(Pro N) antibody. The other was raised against the C-terminal 15-amino acid-residue sequence (Pro C; DMTRISAEWGEFIKK) of the prosegment and termed anti-(Pro C) antibody. The titres of anti-(Pro N) and anti-(Pro C) antibodies were 1:3000 and 1:5000 respectively, which were determined by binding to 50% of 125 I-labelled Tyr-Pro N and 125 I-labelled Pro C peptides respectively.

Antiserum against rat mature renin [anti-(mature renin) antibody] was produced by immunizing rabbits with pure rat renal active renin [15].

Immunoblot analysis

SDS/PAGE was performed on a 5–20% gradient polyacrylamide gel (Atto Co., Tokyo, Japan), according to the method of Laemmli [16]. The proteins, separated by SDS/PAGE, were electrophoretically transferred to a nitrocellulose membrane according to the method of Towbin *et al.* [17]. These sheets were then equilibrated with 0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.5, containing 0.05% (v/v) Tween 20 (T-PBS) and incubated separately with anti-(mature renin), anti-(Pro N) and anti-(Pro C) antibodies diluted 1:10 with T-PBS in the presence of 2% (w/v) BSA for 1 h at room temperature. After being washed, the immunocomplex on these sheets was detected by using a Vecstatin ABC kit, according to the manufacturer's instructions. In brief, these sheets were incubated with biotinylated anti-(rabbit IgG) serum in T-PBS for 30 min, then incubated with avidin-DH-biotinylated horseradish peroxidase H complex for 30 min. After being washed with PBS, the immunocomplex was detected with 0.6 mg of 4-chloro-1-naphthol/ml and 0.015% (v/v) H_2O_2 .

Identification of plasma inactive renin as prorenin

The sample (135 μ l) of plasma inactive renin (0.63 ng of AI/h) partially purified by G3000SW h.p.l.c. was incubated with 15 μ l of anti-(Pro N) antibody or anti-(Pro C) antibody, or pre-immunized serum as a control, for 18 h at 4 °C in 0.1 M-sodium phosphate buffer, pH 7.4, containing 0.15 M-NaCl, 1 mM-EDTA and 1% (w/v) BSA. Following the incubation, 30 μ l of protein A-cellulofine suspension (gel/buffer ratio 1:1), the amount of which was sufficient to precipitate the included IgG, was added and another incubation was carried out with gentle agitation at 4 °C for 24 h. After centrifugation, the residual inactive renin in the supernatant was measured after activation with trypsin, as described above.

To examine whether the trypsin-induced AI-generating activity was due to true renin, a sample of trypsin-treated inactive renin was incubated for 24 h at 4 °C with anti-(mature renin) antibody, or pre-immunized serum as a control; the residual renin activity was then measured.

Affinity for a Cibacron Blue column

Binding to Cibacron Blue was tested by application to a Cibacron Blue-Sepharose column previously equilibrated with 20 mM-sodium phosphate buffer, pH 6.5, containing 1 mM-EDTA, according to the method of Takii *et al.* [18]. Purified recombinant rat prorenin (1 ml, 220 ng of AI/h) and partially purified plasma inactive renin (3.2 ml, 9.7 ng of AI/h) in the equilibration buffer containing 0.1% (w/v) BSA were applied to a Cibacron Blue-Sepharose column (0.8 cm $\times$ 4 cm). After being washed with 20 ml of the equilibration buffer, the column was eluted with 20 ml of equilibration buffer containing 1.4 M-NaCl. The fractions (2 ml) were collected into plastic tubes to which had been added 220 μ l of the equilibration buffer containing 0.2% (w/v) BSA (final concentration). Renin activity in each fraction before and after trypsin treatment was measured, as described above.

H-77-Sephare affinity chromatography

The affinity of trypsin-activated or unactivated recombinant prorenin and plasma inactive renin for an octapeptide renin inhibitor (H-77)-Sephare affinity column was studied [19]. H-77 was coupled to activated 6-aminohexanoic acid-Sephare according to the method of McIntyre *et al.* [20]. The samples of purified recombinant prorenin (270 ng of AI/h) and plasma inactive renin (8.1 ng of AI/h) before and after trypsin treatment were applied to a H-77-Sephare column (0.56 cm $\times$ 1.2 cm) equilibrated with 0.1 M-sodium phosphate buffer, pH 7.4, containing 3 mM-EDTA and 0.1% (w/v) BSA. After being washed with 3 ml of equilibration buffer and then 50 mM-sodium phosphate buffer, pH 7.4, containing 0.35 M-NaCl, 3 mM-EDTA and 0.1% (w/v) BSA, the column was eluted with 3 ml of 0.1 M-acetic acid, pH 2.9, containing 0.15 M-NaCl, 3 mM-EDTA and 0.1% (w/v) BSA. The fractions (0.3 ml) were collected and immediately adjusted to pH 7.4 with 0.2 M-Tris.

Determination of molecular mass

The molecular mass of recombinant prorenin and plasma inactive renin was determined by gel-filtration h.p.l.c. on G3000SW [15]. The column was calibrated with the following molecular-mass standards (Boehringer Mannheim) (Da): aldolase (158 000), BSA (68 000), ovalbumin (45 000), α -chymotrypsinogen (25 000) and cytochrome *c* (12 500).

Determination of optimum pH

The pH-dependence of the AI-forming activity of trypsin-activated recombinant prorenin and plasma inactive renin from rat renin substrate was determined by the method of Figueiredo *et al.* [21].

RESULTS

Purification of recombinant rat prorenin

The renin activity in the serum-free culture medium before and after trypsin treatment was 120 ng of AI/h per ml and 18.3 μ g of AI/h per ml respectively, thereby indicating that prorenin expressed by Chinese-hamster ovary cells was mostly secreted without conversion into mature renin. As shown in Fig. 2(a), most of the protein in the culture medium was not retained by a concanavalin A-Sephare affinity column, whereas about 50% of the recombinant prorenin applied was bound to the column and eluted with 0.2 M-methyl α -mannoside. Gel-permeation h.p.l.c. of prorenin, eluted with 0.2 M-methyl α -mannoside from concanavalin A-Sephare, showed that prorenin was eluted with a retention time of 36.0 min, corresponding to a molecular mass of 46 000 Da, and there was a further separation from other proteins (Fig. 2b). From 100 ml of culture medium, purified prorenin with a total activity of 293 μ g of AI/h was obtained. The overall recovery of prorenin was 16%. The purification factor could not be determined because BSA had been added to the prorenin preparation, a necessary procedure to stabilize the low concentration of purified proteins.

Trypsin activation of recombinant prorenin

The activation of purified recombinant prorenin with trypsin, under optimal conditions, yielded a total renin activity of 583 μ g of AI/h per ml, and the intrinsic activity (activity before trypsin treatment) was 5.25 μ g of AI/h per ml (0.9% of total activity), thereby indicating that the purified prorenin preparation was practically inactive. In the presence of 0.2% (w/v) BSA, the intrinsic activity of prorenin did not increase after storage at -30°C for 1 month.

Fig. 2. Concanavalin A-Sephare affinity chromatography (a) and gel-permeation h.p.l.c. (b) of recombinant prorenin

(a) Concanavalin A-Sephare chromatography of culture medium separated prorenin into two forms, including the concanavalin A-non-bound form and the bound form eluted with 0.2 M-methyl α -mannoside (α -MM). The bound form of prorenin was pooled for further purification, as indicated by the bracket. Renin activity was determined before (○) and after (●) trypsin activation. ----, A_{280} . (b) Prorenin, eluted with 0.2 M-methyl α -mannoside from a concanavalin A-Sephare column, was applied to a G3000SW column equilibrated with 50 mM-sodium phosphate buffer, pH 7.4, containing 0.25 M-NaCl and 5 mM-EDTA and eluted with the equilibration buffer. The bracket indicates the fractions that were pooled as purified recombinant rat prorenin. Renin activity was determined before (○) and after (●) trypsin activation. ----, A_{280} . Retention times of protein standards, comprising aldolase (158 kDa), BSA (68 kDa), ovalbumin (45 kDa) and α -chymotrypsinogen (25 kDa), are indicated by arrows. V_0 , void volume.

Partial purification of rat plasma inactive renin by h.p.l.c.

Inactive renin was eluted with a retention time of 36.5 min, corresponding to a molecular mass of 48 000 Da, a larger mass than that of active renin (40 000 Da) (results not shown). H.p.l.c. was carried out ten times and fractions of inactive renin were pooled (total activity 3.03 ng of AI/h per ml; active renin, 0.24 ng of AI/h per ml), for further characterization.

Immunoblot analysis

As shown by immunoblot analysis following SDS/PAGE in Fig. 3, purified recombinant prorenin showed a single band with a molecular mass of 42 000 Da, which was immunoreactive to anti-(mature renin), anti-(Pro N) and anti-(Pro C) antibodies

Fig. 3. Immunoblot analysis of recombinant prorenin and pure renal active renin

The samples of trypsin-treated or untreated pure renal active renin and recombinant prorenin were subjected to SDS/PAGE and electrophoretically transferred to the nitrocellulose membrane. The membrane was incubated with anti-(mature renin) antibody, anti-(Pro C) antibody or anti-(Pro N) antibody for the detection of immunoreactive protein. Lanes 1, pure rat renal active renin; lanes 2, purified recombinant prorenin; lanes 3, recombinant prorenin activated by incubation with trypsin (78 μ g/ml) for 30 min at 4 °C; lanes 4, pure rat renal active renin incubated with trypsin in the same condition as the case of prorenin in lanes 3.

(lanes 2). Pure rat renal active renin showed a single band with a molecular mass of 36 000 Da, which was recognized by anti-(mature renin) antibody but by neither anti-(Pro N) antibody nor anti-(Pro C) antibody (lanes 1).

Activation of recombinant prorenin with trypsin increased the renin activity 110-fold and decreased the molecular mass from 42 000 to 32 000 Da, which was detected with anti-(mature renin) antibody (Fig. 3, lanes 3). This was accompanied by loss of the immunoreactivity to both anti-(Pro N) and anti-(Pro C) antibodies (lanes 3). These results show that trypsin removes the prosegment from prorenin.

Trypsin treatment of rat renal active renin decreased the molecular mass from 36 000 to 32 000 Da (lanes 4), the same value as for the trypsin-activated recombinant prorenin. This was associated with a 62.5% decrease in renin activity.

Identification of plasma inactive renin as prorenin

The immunological characteristics of partially purified plasma inactive renin were studied by using the immunoprecipitation method. Anti-(Pro C) and anti-(Pro N) antibodies immunoprecipitated 90% and 72% respectively of inactive renin obtained from G3000SW h.p.l.c. The AI-generating activity of trypsin-activated inactive renin was inhibited by prior incubation with anti-(mature renin) serum by 90%. These results indicate that inactive renin was true prorenin.

Cibacron Blue–Sephacel affinity chromatography

Both recombinant prorenin and native inactive renin from plasma were bound to a Cibacron Blue–Sephacel column and eluted with 1.4 M-NaCl. For both these samples, pretreatment with trypsin caused loss of affinity for the column (results not shown).

H-77–Sephacel affinity chromatography

Neither recombinant prorenin nor inactive renin from plasma had affinity for an H-77–Sephacel affinity column, whereas both trypsin-activated recombinant prorenin and native inactive

Fig. 4. pH-dependence of AI-generating activity of trypsin-activated recombinant prorenin (a) and plasma inactive renin (b)

Each value represents the mean $\pm$ S.E.M. ($n = 3$).

renin were retained by this column and eluted at 0.1 M-acetic acid, pH 2.9 (results not shown).

Optimum pH

The pH-dependence for AI-generating activity from rat renin substrate was similar for recombinant prorenin and plasma inactive renin (Fig. 4). The optimum pH was 7.5 and 7.0 for trypsin-activated recombinant prorenin and plasma inactive renin respectively.

DISCUSSION

We have obtained evidence that recombinant rat prorenin is similar to rat plasma inactive renin, with respect to physicochemical characteristics, indicating that rat plasma inactive renin is prorenin. Thus the availability of recombinant rat prorenin will pave the way for studies on the physiological significance of prorenin.

In humans, most of the renin in plasma is prorenin and is enzymically inactive [2–6]. However, studies on the physiological role of circulating prorenin have been hampered by the difficulty in purifying sufficient amounts of native human prorenin [22]. Prorenin is not stable during the purification procedure and concentrations of this protein in the plasma and kidney are low [23].

The availability of recombinant cDNA technology made feasible the preparation of recombinant human prorenin, a

Table 1. Physicochemical characteristics of recombinant rat prorenin and plasma inactive renin

	Recombinant prorenin	Plasma inactive renin
Immunoreactivity		
Anti-(mature renin) antibody	+	+
Anti-(Pro N) antibody	+	+
Anti-(Pro C) antibody	+	+
Binding to Cibacron Blue	+	+
Binding to H-77		
Before activation	—	—
After activation	+	+
Optimum pH	7.5	7.0
Molecular mass		
By h.p.l.c.	46000 Da	480 000 Da
By SDS/PAGE	42000 Da	Not determined

substance biochemically similar to the native protein, by Chinese-hamster ovary cells transfected with the preprorenin cDNA [7,11,24,25]. Using pure recombinant human prorenin labelled with ^{125}I , we found that prorenin is not activated in the blood but that rather the activation occurs in the liver and kidney, suggesting the contribution of prorenin to the generation of angiotensin in local tissues [8]. Using recombinant human prorenin as the substrate, Shinagawa *et al.* noted the presence of the enzyme that processes prorenin to mature renin in the human kidney [26]. Thus the recombinant human prorenin preparation is a useful tool for elucidation of the physiological significance of prorenin. However, further study requires the availability of a suitable animal model.

In the present study, purified recombinant rat prorenin cross-reacted with both anti-(Pro N) and anti-(Pro C) antibodies. The molecular mass, estimated by SDS/PAGE (Fig. 3) and h.p.l.c. on G3000SW (Fig. 2b), was similar to that calculated from the predicted amino acid sequence of rat prorenin from the renin gene [9]. These observations suggest that this prorenin is intact zymogen, not truncated in the prosegment portion, although verification will require an N-terminal sequence analysis. Furthermore, rat recombinant prorenin had no affinity for a renin inhibitor-Sepharose column, but was retained by a Cibacron Blue-Sepharose column. These characteristics are consistent with those of human recombinant prorenin [7,27].

In contrast with the identification of inactive renin as prorenin in humans, it was unclear whether inactive renin in rats was indeed prorenin [28–30]. In the present study, using immunological techniques, we have found that rat plasma inactive renin is indeed prorenin. Furthermore native prorenin was found to be much the same as recombinant prorenin with respect to physicochemical characteristics (Table 1).

Of interest is the observation that the activation of recombinant prorenin with trypsin decreased the molecular mass from 42000 to 32000 Da, the same value as that of trypsin-treated pure renal active renin (Fig. 3). These findings indicate that trypsin treatment led to cleavage of a part of the mature renin sequence as well as that of the prosequence. This may explain why the optimum pH of trypsin-activated recombinant prorenin and native prorenin showed a slightly higher value (Fig. 4) than that of pure renal active renin (pH 6.5) reported by Figueiredo *et al.* [21] and by us [12].

In conclusion, recombinant rat prorenin and native inactive renin show striking biochemical similarities, indicating that rat plasma inactive renin is prorenin.

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