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Binding properties of rat prorenin and renin to the recombinant rat renin/prorenin receptor prepared by a baculovirus expression system

A.H.M. NURUN NABI[1], AYANO KAGESHIMA[3], MOHAMMAD NASIR UDDIN[2], TSUTOMU NAKAGAWA[2], ENOCH Y. PARK[2,3] and FUMIAKI SUZUKI[1,2]

[1]Graduate School of Agricultural Science and [2]Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, [3]Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Shizuoka 422-8529, Japan

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Abstract. The rat recombinant renin/prorenin receptor (AB188298 in DDJB), which conjugated with FLAG epitope in its N-terminus, was expressed in a baculovirus expression system. The recombinant receptor, prepared from the cytoplasmic fraction of the insect cells, was identified by Western blotting using anti-FLAG antibody. Prorenin as well as renin bound to the receptor with different binding affinities. Their K_d values were estimated at 8.0 and 20 nM, respectively. The amounts of prorenin and renin bound to the immobilized receptors were 1.0 and 0.2 pmole, respectively. The prorenin bound to the receptor had renin activity and the renin kept the activity at similar level to that before the binding. The K_m of their complexes was the same at 3.3 μM when sheep angiotensinogen was used as the substrate. Their V_{max} values were 5.5 and 10 nM·h^{-1}, respectively. The molecular activities of prorenin and renin bound to the receptor were 1.1 and 10 h^{-1}, respectively. From these findings, rat prorenin as well as renin was indicated to bind to the recombinant receptor and express the enzymatic activity *in vitro*.

Introduction

Renin-angiotensin system plays a pivotal role in maintaining the blood pressure and electrolyte balance in mammals (1). Recently, prorenin, the inactive proenzyme of renin, was studied in relation to the system. Prorenin with 43 amino acid residue in the prosegment region has reportedly been activated non-proteolytically by an acidification or under low temperature to change its conformation locally (2,3). A functional renin/prorenin receptor has been found that acts as a renin/prorenin cofactor on the cell surface, enhancing the efficiency of angiotensinogen cleavage by renin and unmasking prorenin catalytic activity (4). Moreover, the non-proteolytic activation of prorenin through protein-protein interaction has been demonstrated *in vitro* at physiological pH, using specific antibodies (5).

Many kinds of renin/prorenin receptors have been reported (6-8). For example, the mannose-6-phosphate receptor, which has been the most extensively studied, mediated only uptake of renin and prorenin in the cell rather than showed other functions on the cell membrane (6). On the other hand, prourokinase binding to the urokinase receptor was reported to induce a conformational change in the proform into the non-proteolytic activation (9). Certain receptor-bound mature enzymes were much more efficient in the catalytic activity (10,11). We have transiently expressed the rat renin/prorenin receptor on the membrane of the COS-7 cells to study the non-proteolytic activation mechanism of the rat prorenin (12). The expressed amount on the membrane was insufficient to investigate the binding mechanism in detail. In this study, a baculovirus expression system (13,14) was applied to obtain sufficient amount of recombinant rat renin/prorenin receptor.

As several strains of rats are widely used as a model animal in the studies on blood pressure regulation and hypertension, rat prorenin reports have attracted the attention of renin and prorenin investigators. However, the activation of rat prorenin took longer at acidic pH (15) and the rate of its acid activation was one-fifth of that of the human prorenin (16). The rat prorenin has commonly been considered inactive in blood circulation. In this study, we investigated *in vitro* the binding properties of rat renin and prorenin to the recombinant renin/prorenin receptor, obtained by the baculovirus expression system, to understand the binding and activation of rat renin and prorenin at a neutral pH.

Materials and methods

Antibodies. Different types of antibodies were used in this study. Anti-rat renin antibody (a kind gift from Professor Inagami, 17) was used to detect rat mature renin. MC13

Correspondence to: Dr Fumiaki Suzuki, Laboratory of Animal Biochemistry, Faculty of Applied Biological Sciences, Gifu University, Yanagido 1-1, 501-1193 Gifu, Japan
E-mail: aob3073@cc.gifu-u.ac.jp

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antibody against the C-terminal region of the prosegment of prorenin was prepared in immunized rabbit. Anti-FLAG-M2 antibody, Protein A-HRP (horseradish peroxidase) conjugate and anti-mouse IgG antibody labeled with HRP were purchased from Sigma and Bio-Rad, USA.

Construction of the vector. The rat renin/prorenin receptor cDNA with FLAG epitope was amplified by PCR using *Pst*I and *Eco*RI restriction sites. The designed primers were as follows: forward primer: 5'-CACCATGGCTGTTGTCGT CTCCTC-3'; reverse primer: 5'-CGGAATTCTAATCCA TTCGAATCTTCTGG-3'. The PCR product was inserted into the entry vector, pENTR/D-TOPO (Invitrogen, San Diego, CA, USA) containing *Pst*I and *Eco*RI restriction sites along with rat signal sequence and cytoplasmic region. The resulting pENTR-renin/prorenin receptor-FLAG, was inserted into a pDEST 8 donor vector by Gateway Cloning Technology (Invitrogen). The recombinant donor vector, pDEST-renin/ prorenin-FLAG was transformed into *E. coli* DH10, which contained a bacmid. The recombinant bacmid was extracted from *E. coli* and transfected Tn-5B1-4 cells from *Trichoplusia ni* (Invitrogen). Finally, the recombinant AcNPV-renin/ prorenin receptor cDNA harboring plasmid under the control of viral polyhedron promoter was constructed. Four million Tn-5B1-4 cells were infected at a multiplicity of infection (MOI) of 5 with a recombinant baculovirus. The cytoplasmic fraction (12.0 ml) of the receptor was collected two days after transfection of the cells.

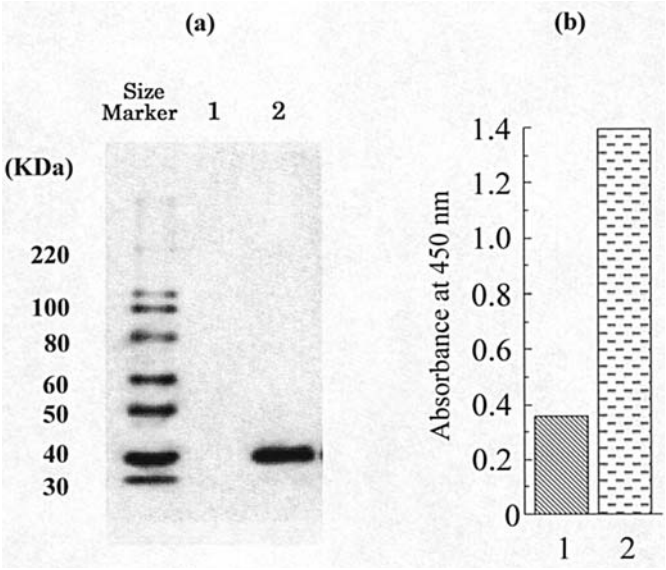

Figure 1. Western blot analysis of the cytoplasmic fraction from Tn5 cells. Protein band was detected by mouse anti-FLAG-M2 monoclonal antibody and anti-mouse IgG antibody labelled with HRP. Molecular mass of the recombinant rat renin/prorenin receptor was 40 kDa as shown in lane 2 (a) and lane 1 was for the fraction of control cells without any transfection. Recombinant receptors immobilized in the 96-well plates were confirmed by using the anti-FLAG-M2 antibody and Protein A-HRP conjugate as shown by bar 2 (b). Cytoplasmic fraction of the cells without any transfection was used as the control shown by bar 1 (b).

Western blot analysis. To detect the expression of recombinant protein, cytoplasmic fractions were subjected to SDS/PAGE (12% polyacrylamide) (18) using the Mini-protean II system (Bio-Rad). Total protein of culture supernatants was precipitated with 2% (w/v) trichloroacetic acid and washed with 50 mM Tris/HCl (pH 7.5). Precipitated protein was dissolved with SDS/PAGE sample buffer (9.5 ml) [3.5 ml of distilled water, 1.25 ml of 0.5 M Tris/HCl (pH 6.8), 2.5 ml of glycerol, 2 ml of 10% (w/v) SDS and 0.2 ml of 0.5% (w/v) bromophenol blue] and subjected to SDS/PAGE. Magic Mark™ XP Western protein standards (Invitrogen) were used as standard protein for the blot and using anti-FLAG-M2 and anti-mouse IgG/HRP (horseradish peroxidase) antibodies the bands were visualized. After SDS/PAGE, the proteins were blotted onto a PVDF membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). After blocking in 5% Tris-buffered saline (pH 7.6) [2.42 g (20 mM) Tris-base, 8 g NaCl and 3.8 ml (1 M) HCl] containing 0.1% Tween-20, the membrane was incubated in 1:10,000 diluted anti-FLAG-M2 solution for 1 h. The membrane was washed and then incubated in 1:20,000 diluted anti-mouse IgG antibody labeled with HRP for 1 h. Using ECL plus Western blotting reagent pack (Amersham Biosciences) specific bands were detected. Those bands were analyzed using a Fluor-S/MAX multi-imager (Bio-Rad).

Preparation of rat renin and prorenin. Chinese hamster ovary cell line harboring rat prorenin cDNA was maintained (19) under humidified atmosphere of 5% CO_2 and 95% air in 25-cm^2 cell culture flasks (Cellstar, Greiner, Bio-One Germany) until achieving 100% confluent monolayer in the Dulbecco's modified Eagle's medium (DMEM) containing 0.1 mM non-essential amino acids, 2 mM glutamine, 100 U penicillin, 100 μg streptomycin per ml, 200 nM methotraxate supplemented with 5% dialyzed FBS. Recombinant rat renin (Mr: 42,000) was prepared by the proteolytic activation of recombinant rat prorenin (M_r 46,000) with trypsin at 25˚C for 40 min (final concentration was 2.0 mg/ml). Immobilized Soybean Trypsin Inhibitor (agarose-bound SBTI, 4.0 mg/ml) was added to stop the proteolytic reaction. Finally, the reaction mixture was centrifuged twice with immobilized SBTI to remove any trace of trypsin. The prorenin and renin preparations were stored at -80˚C until further study.

Determination of K_d for receptor binding of rat renin and prorenin. After 20 times dilution of the original receptor preparation, 200 μl aliquots were allowed to immobilize in the 96-well plate at 4˚C for 24 h using blocking buffer (0.1% casein in phospate-buffered saline). The prorenin preparation was incubated at 37˚C for 1 h to avoid possible cryo-activation. By this treatment, variation in the inactive prorenin level in each experiment was minimized to <2% of its total potential renin activity attainable by trypsin treatment. The K_d values for the binding of renin and prorenin to the receptor were calculated by determining the concentration-dependent binding to the receptor. Different concentrations of renin and prorenin (from 1 to 20 nM) incubated in the 200 μl medium with the immobilized recombinant receptors at 4˚C for 30 min. After incubation, renin and prorenin media were removed. Amounts of receptor-bound renin and prorenin were estimated by subtracting remained amount of renin and prorenin from their initial concentration.

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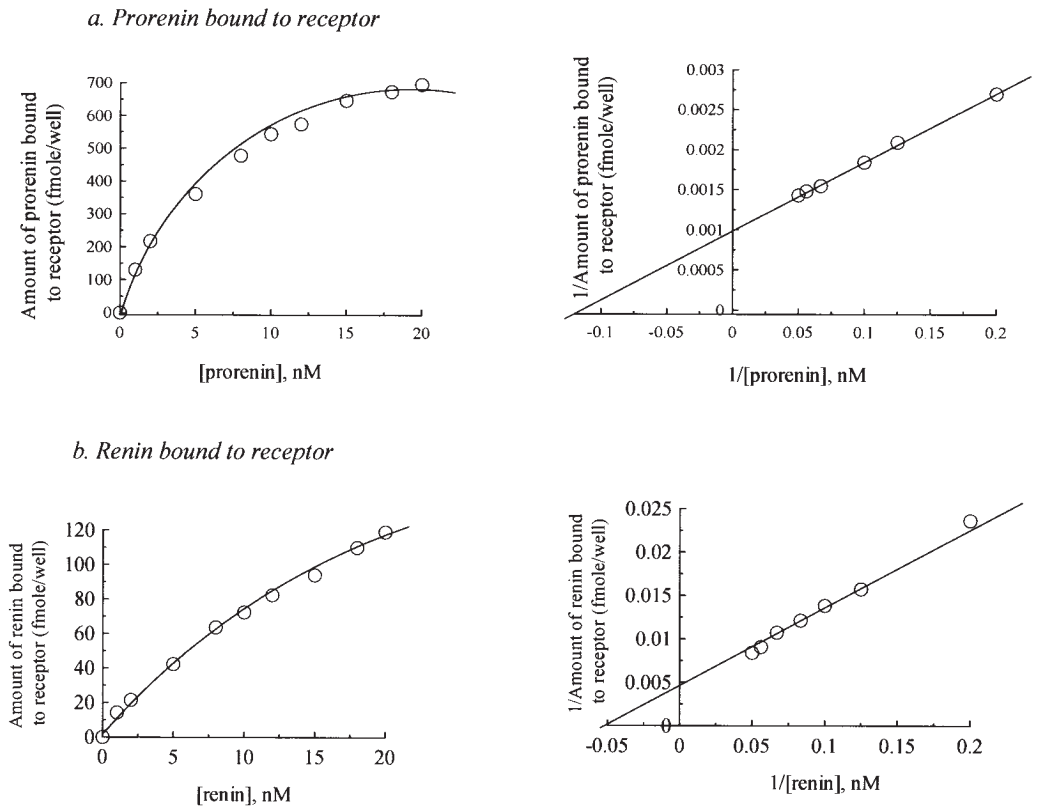

Figure 2. Binding of rat prorenin and renin to the rat renin/prorenin receptor. Different concentrations (1-20 nM) of renin and prorenin were allowed to bind with the recombinant immobilized receptor. Amounts of bound renin and prorenin (200 μl of the original preparations) were determined by subtracting the remaining amount of unbound renin/prorenin from their initial amount. The double reciprocal plots, 1/amount of receptor-bound prorenin vs. 1/[prorenin] (a) and 1/amount of receptor-bound renin vs. 1/[renin] (b), were used to determine the K_d and maximum binding amounts. The K_d values were estimated to be 8.3 and 20 nM, respectively. The maximum binding amounts of renin and prorenin were 0.2 and 1.0 pmole, respectively.

Determination of K_m of the receptor-bound renin and prorenin. Rat renin and prorenin preparations were incubated in 200 μl of medium with the receptors coated in the 96-well plate at 4˚C for 30 min and then removed. The wells were washed with ice-cold PBS. The K_m values were determined from the rate of Ang I production at sheep angiotensinogen concentrations of 0.3-2.8 μM. Enzymatic activities of receptor-bound renin and prorenin were measured by angiotensin I ELISA (20) after incubation of receptor-bound renin and prorenin with the recombinant sheep angiotensinogen preparation (21) under standard assay conditions as described previously (22).

Estimation of the molecular activity of the bound form of renin and prorenin. Different concentrations of renin and prorenin (from 1 to 20 nM) were incubated in the 200 μl medium with the recombinant receptors immobilized in the 96-well plate at 4˚C for 30 min. After incubation, amounts of receptor-bound renin and prorenin were estimated by subtracting the remaining amount of renin and prorenin from their initial concentration under standard assay conditions. Double reciprocal plots were constructed to determine the maximum binding amount of rat renin and prorenin to the immobilized recombinant receptors. Finally, molecular activities of receptor-bound renin and prorenin were calculated by dividing V_{max} values by their respective maximum binding amounts. For the soluble form of mature renin, molecular activity was estimated by dividing V_{max} value by the renin concentration used (10 nM).

Results

Western blot analysis of the recombinant receptor. The recombinant rat renin/prorenin receptor from the cytoplasmic fraction of the insect cells migrated to the 40,000 region after Western blotting using anti-FLAG-M2 and anti-mouse IgG/HRP antibodies (Fig. 1a). The receptor preparation was allowed to immobilize in the wells of the 96-well plate, and these immobilized receptors were recognized by the anti-FLAG (Fig. 1b) antibody.

Binding assay. In the case of rat renin and prorenin bound to the immobilized recombinant receptor, the absorbance was measured 0.631 and 0.621, respectively at 450 nm under standard assay conditions. On the other hand, their respective blank values were 0.191 and 0.184. The K_d values for the renin and prorenin binding to the rat renin/prorenin receptor were estimated at 8.3 and 20 nM, respectively (Fig. 2a and b). In each well, the amount of the immobilized recombinant receptor was calculated at ~4.2 pmole from the K_d value. The maximum binding amounts of renin and prorenin were 0.2 and 1.0 pmole, respectively (Fig. 2a and b).

Molecular activities of the soluble phase and receptor-bound renin and prorenin. The molecular activities of the receptor-bound renin and prorenin were 10 and 1.1 h^{-1}, respectively. The molecular activity of renin in the soluble phase was 1.25 h^{-1}.

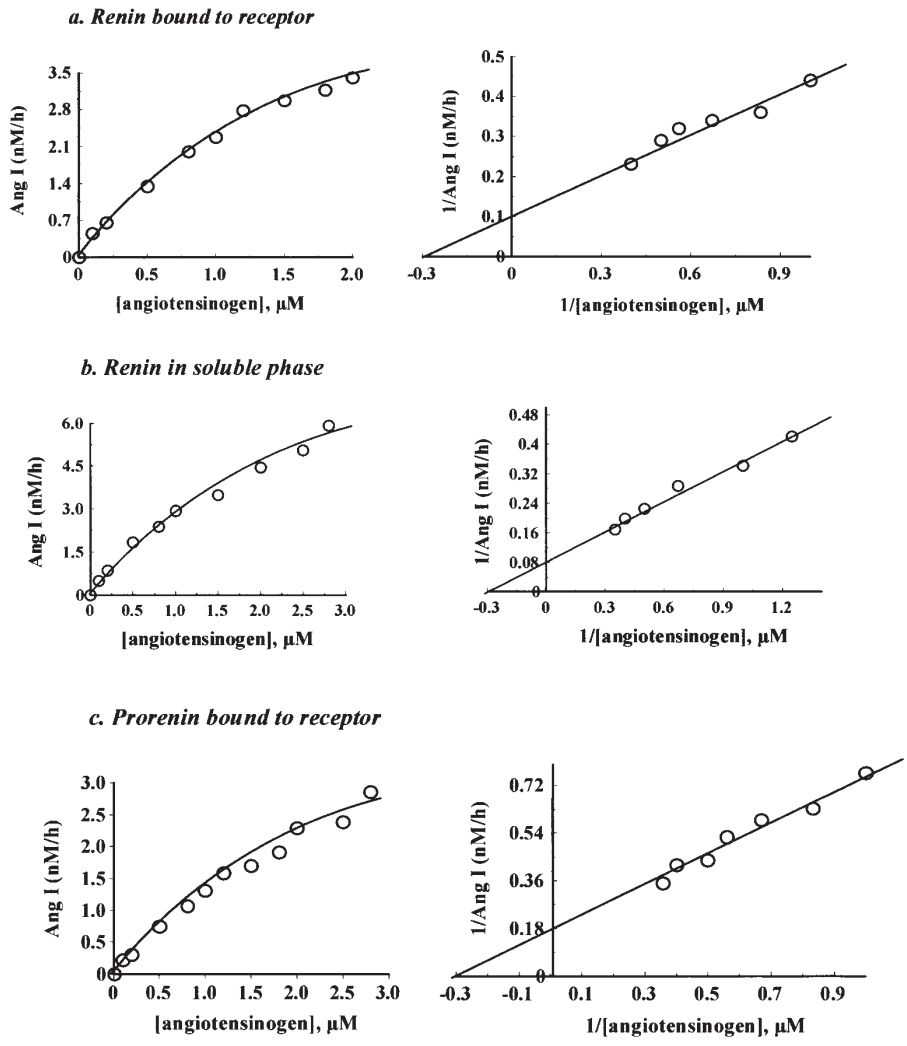

Figure 3. The Ang I generation rate by soluble phase and receptor-bound renin and prorenin. (a and b) Lineweaver-Burk plots of 1/Ang I/ h vs. 1/angiotensinogen by renin bound to the receptors and in soluble phase (10 nM), respectively. The K_m value was similar for both the cases estimated at 3.33 μM while the V_{max} values determined from these plots were 10 nM·h^{-1} and 12.5 nM·h^{-1}, respectively. (c) The Ang I generation rate by receptor-bound rat prorenin. The K_m and V_{max} values determined from the double reciprocal plot (1/Ang I generated/h vs. 1/angiotensinogen) were 3.33 μM and 5.5 nM h^{-1}, respectively. Rat renin and prorenin (10 nM, 200 μl) were applied into each well containing immobilized receptors for each experiment.

The K_m of receptor-bound rat renin and prorenin. Receptor-bound renin and prorenin showed their enzymatic activities by cleaving sheep angiotensinogen. The K_m values of the receptor-bound renin and prorenin using sheep angiotensinogen were similar at 3.33 μM (Fig. 3a and c). The K_m value for the renin in soluble phase was also 3.33 μM using the same substrate (Fig. 3b).

Discussion

We expressed rat renin/prorenin receptor by a baculovirus expression system to investigate biochemical properties of the receptor for *in vitro* binding to rat renin and prorenin. The receptor preparation obtained from the cytoplasmic fractions of insect cell transformants was confirmed by Western blotting using the antibody against the FLAG epitope. Its molecular weight was 40 kDa (Fig. 1a) that was identical to that of human renin/prorenin receptor expressed in the COS-7 and human mesangial cells as reported by Nguyen *et al* (11). The receptors immobilized in the wells of the 96-well plate were also recognized by the anti-FLAG-M2 antibody conjugated with Protein A-HRP (Fig. 1b). The binding of renin and prorenin to the receptor could be specifically observed by antibodies either against rat mature renin region or the C-terminal region of rat prorenin prosegment.

Using sufficient amount of rat renin/prorenin receptor, we elucidated the binding properties of renin and prorenin to the common receptor to propose a possible binding mechanisms as shown in Fig. 4. We found that the K_d value for the binding of prorenin with the receptor was 8.3 nM whereas this value for the renin was almost 2.5 times higher, K_d = 20 nM (Fig. 2a and b) indicating that prorenin prosegment plays a specific role in the prorenin-receptor binding. Suzuki *et al* (5) showed *in vitro* using region-specific antibodies that human prorenin has a 'handle' region in the prosegment sequence of prorenin molecule, and predicted that such a region played a key role in the binding with specific proteins (5,23). The recombinant receptor has a 'high affinity region' as indicated by closed

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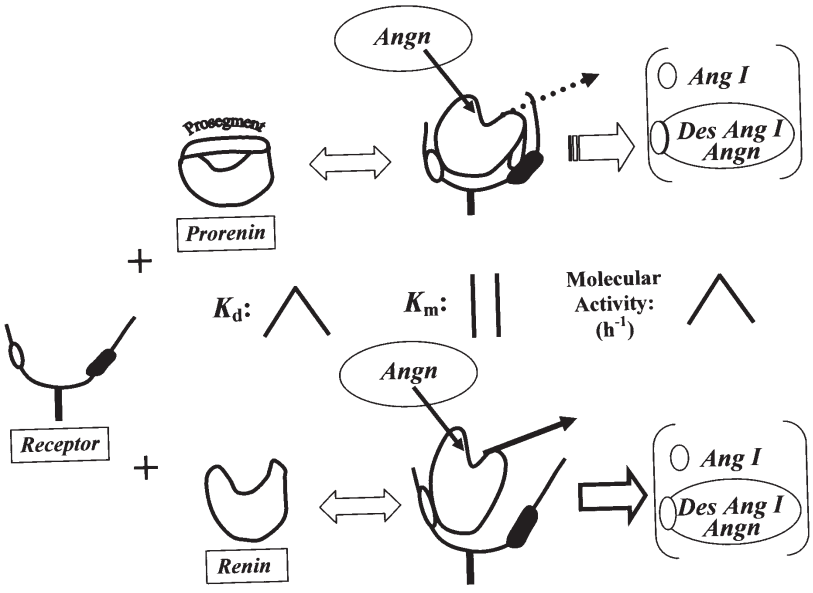

Figure 4. Possible binding mechanisms of renin and prorenin to the receptor. Each of the reactions was carried out at pH 6.5 to 7.5. In the receptor, the associated regions specific to prorenin and common for renin as well as prorenin are indicated by the closed and open symbols, respectively.

symbols in Fig. 4, which could recognize specifically the 'handle' peptide of prosegment sequence of prorenin.

The same K_m value at 3.33 μM for both the receptor-bound renin and prorenin (Fig. 3b and c) indicated the association of these two molecules with the receptor did not hinder making the complex of enzyme-substrate, prorenin/renin-angiotensinogen, in the enzymatic level. The molecular activity of the receptor-bound renin was almost 10 times higher than that of prorenin. This is probably due to the change in the conformation of prorenin molecule by the association to the receptor, which ultimately delays the release of the products, angiotensin I and des-Ang I-angiotensinogen, as shown in Fig. 4.

We observed that rat prorenin was non-proteolytically activated by binding to the receptor within a short period (in minutes). This is the first finding that rat prorenin can be activated at pH 6.5, because such activation has been reported only on observation under low pH and long time period (in days) (15,16).

Rat renin bound to the receptor had 8 times higher molecular activity than that in the soluble phase, although the K_m was the same 3.3 μM, as shown in Fig. 3a and b. The turnover rate of the products from the complex of immobilized enzyme and substrate is higher than that of the free form of mature renin and substrate. This observation is similar to the data reported by Nguyen *et al* (11) that receptor-bound human renin could generate Ang I from human angiotensinogen 4-5 times more efficiently compared to renin in solution. This is probably due to the cleft space of the renin molecule that was more altered under the complex with the receptor.

In this study, we have shown the physiological importance of renin/prorenin receptor and its probable role associated with the renin-angiotensin system, particularly with the local renin-angiotensin system on the cell membrane *in vivo*. Further study will elucidate other roles of the prosegment sequence of prorenin in this binding and the possibility of its association in signal transduction.

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