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Prorenin has high affinity multiple binding sites for (pro)renin receptor

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ABSTRACT

An important role of the decoy peptide sequence has recently been suggested *in vitro* for the binding of prorenin to the (pro)renin receptor [(P)RR]. In this study, other prospective crucial regions in renin and prorenin responsible for their interaction with (P)RR were investigated using various kinds of peptides, e.g., the "hinge" S^{149}QGVLKEDVF^{158} designed from the structure of renin also common to prorenin, L^{1P}PTDTTTF^{8P}, L^{1P}PTDTTTFKRIFLKR^{15P} and the decoy (R^{10P}IFLKRMPSI^{19P}) designed from the predicted structure of prorenin. For the kinetic analysis, the recombinant h(P)RR was immobilized on the biosensor surface through a specific anti-(P)RR antibody. In case of the equilibrium state analysis, the (P)RR was directly adsorbed on plastic wells for observing the bindings of renin/prorenin. The dissociation constants (K_D) for the bindings of renin and prorenin to the pre-adsorbed receptors were 4.5 and 1.0 nM, respectively, similar to those stated in the kinetic study by BIAcore assay. The "hinge" region peptide bound to (P)RR in a dose-dependent manner with a K_D estimated 17.0 nM which was five times higher than that of the decoy. The K_D values for L^{1P}PTDTTTF^{8P} and L^{1P}PTDTTTFKRIFLKR^{15P} were 52 and 7.6 nM, respectively. The "hinge" peptide, as the decoy, inhibited the bindings of renin and prorenin to (P)RR. The inhibition constant (K_i) for the binding of renin and prorenin by the decoy and "hinge" were 16.7 and 15.1, and 37.1 and 30.7 nM, respectively. These *in vitro* studies suggest that renin has a single and prorenin has at least two high affinity binding sites for the (P)RR.

1. Introduction

Human (pro)renin receptor [h(P)RR] with a single transmembrane domain was cloned by Nguyen et al. [1] that binds to renin and prorenin. The bindings of renin and prorenin have been confirmed using COS-7 cells [2], vascular smooth muscle cells [3] expressing h(P)RR on their membrane and immobilized recombinant rat (P)RR [4]. These studies have revealed that (P)RR-bound renin displays an increased catalytic activity compared with the free form of mature renin in solution [1,5–7] and (P)RR plays a crucial role in the generation of angiotensin at tissue sites as it is expressed ubiquitously in liver, heart, kidney, testes, ovary [1], brain [8] and retina [9]. Also, receptor-bound human as well as rat prorenin was activated by 30% and 40%, respectively, through conformational change after interaction with the receptor [2,4]. Schefe et al. have demonstrated a novel signal transduction of (P)RR involving the receptor and the promyelocytic zinc finger motif (PLZF) [10]. Both renin and prorenin after binding to the receptor on the cell membrane initiated signal transduction by activating mitogen activated protein (MAP) kinase–extracellular signal regulated kinase (ERK) 1/2 signaling pathway [1,11]. These data showed that receptor-bound renin and prorenin can exert their pathophysiological effects in angiotensin II dependent and independent manner.

The term "handle" region peptide or HRP (I^{11P}FLKR^{15P}) was first coined by Suzuki et al. [12] who demonstrated its crucial role for the interaction of prorenin molecule with its binding proteins. Based on this sequence Ichihara and colleagues [13] designed a 10-amino acid sequence, R^{10P}IFLKRMPSI^{19P}, termed as decoy peptide. Through a substantial number of studies they demonstrated the involvement of (P)RR in the pathogenesis of diverse diseases specially related to end-stage organ damage that could be ameliorated by decoy peptide containing the HRP in wild-type rats or in the transgenic rats over-expressing h(P)RR and human prorenin [13–16]. In these *in vivo* studies, inhibition of prorenin binding to the receptor by the decoy was reported. The functional ability of decoy peptide as a (P)RR blocker was further confirmed by other researchers as well [8,17,18]. In addition, our previous study revealed that the binding affinity of the prorenin for the receptor ($1/K_D$) was three times higher than that of mature renin [4] and a recent study reported that decoy peptide directly bound to the recombinant (pro)renin receptor [19], suggesting a probable pivotal role of the R^{10P}IFLKRMPSI^{19P} sequence in prorenin prosegment for the binding. Moreover, the binding of mature renin lacking prosegment sequence to the receptor still remains

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inexplicable. Hence, it was assumed that there must be a common region both in renin and prorenin that is accountable for their binding to (P)RR. A flexible surface protein has been reported to regulate the function of proteins with bilobal structure [20,21]. Such region localizes in the "hinge" region (S^{149}QGVLKEDVF^{158}) of the mature renin [22] and is present commonly both in renin and prorenin molecules [23]. Using these peptides, we, in this study, investigated the binding properties of renin and prorenin to (P)RR by kinetic study in BIAcore assay system as well as equilibrium study with the adsorbed (P)RR on the plastic surface and thus evaluate the possible binding sites in renin and prorenin for the receptor.

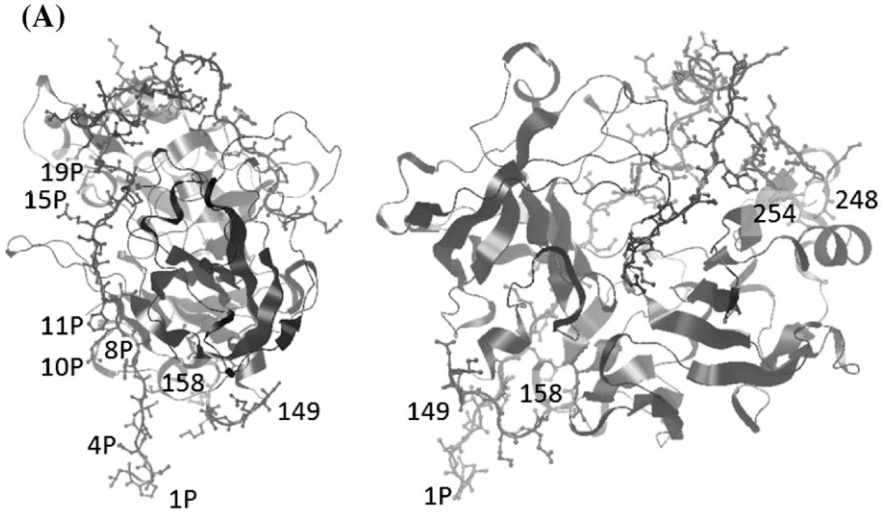
2. Materials and methods

2.1. Designation of peptides

The designation of peptides 1^P-4^P ($L^{1P}PTD^{4P}$), A^{248}KKRLFDYVV^{257} and the decoy (R^{10P}IFLKRMPSI^{19P}) has been pointed out recently [19]. In addition to these, other peptides from different regions of the amino-terminal sequence of prorenin prosegment (Fig. 1), such as 1^P-8^P ($L^{1P}PTDTTTF^{8P}$) and 1^P-15^P ($L^{1P}PTDTTTFKRIFLKR^{15P}$), were designed on the basis of the primary structure of the prosegment and the stereo structure of human prorenin predicted by the homology modeling method (Fig. 1A and B). A sequence, named as "hinge" peptide (S^{149}QGVLKEDVF^{158}, Fig. 1A and C), present both in prorenin and mature renin was designed on the basis of stereo structure of renin as shown in Fig. 1D. These peptides were obtained from Operon, Japan with 98–99% purity. Peptides were dissolved in Milli Q water (1.0 mg/ml). During BIAcore and equilibrium state analyses further dilutions for desired concentrations of the peptides (e.g., 800, 400, 200, 100, 80, 40, 20 and 10 nM) used in the experiments were made using HBS-EP buffer (0.15 M NaCl, 3.0 mM EDTA, 0.005% polysorbate 20 and 0.01 M HEPES, pH 7.4) and PBS, respectively.

2.2. Preparation and purification of recombinant h(P)RR

A cDNA coding for the extracellular part of h(P)RR (containing 292 amino acid residues from N17-N308) with hexa His tag sequence was



(B) Sequence of 1^P-4^P, 1^P-8^P, 1^P-15^P and decoy peptide from prorenin prosegment

Prorenin:**LPTDTTTFKRIFLKRMPSIRESLKERGVDMARLGPEWSQPMKR**-Renin

1^P-4^P
10^P-19^P
Decoy peptide
1^P-8^P
"Handle" region peptide
11^P-15^P
1^P-15^P

(C) Sequence of "hinge" region

^{149}S-Q-G-V-L-K-E-D-V-F^{158}

(D) Sequence in the C-domain

^{248}A-K-K-R-L-F-D-Y-V-V^{257}

Fig. 1. Designation of peptides in the tertiary structure of prorenin predicted by the protein modeling method. (A) Tertiary structure of prorenin predicted by the homology modeling. In this procedure, the primary structures of human prorenin, hog pepsinogen, atomic coordinates of pepsinogen (2PSG [PDB] in Protein Data Bank code) and a module of Insight II were used for the structural data and modeling software, respectively. In both of the figures, the models of wire frame and ribbon indicate regions in the prosegment and renin, respectively. The morphological relationship between the prosegment and renin molecule in the predicted structure was fundamentally the same as described previously [12]. (B) The amino acid sequences of prosegment part of prorenin with decoy including the "handle" region peptide (HRP), 1^P-4^P, 1^P-8^P and 1^P-15^P peptides. (C) "Hinge" region is present both in prorenin and mature renin molecules. (D) Sequence present in the C-domain of mature renin. The predicted tertiary structure of prorenin has been presented in two different angles to show the positions of the peptides within the prosegment sequence.

inserted into the vector, pIVEX 1.3 WG. The receptor (32.5 kDa, the extracellular part) was expressed in a cell free *in vitro* system based on wheat germ lysate using RTS 500 Wheat Germ CECF kit according to the manufacturer's instruction (Roche Diagnostics GmbH, Germany). Thus, the recombinant receptor was purified by affinity chromatography using His Trap column. The proteins eluted by 500 mM imidazole at pH 7.4 were fractionated and aliquots of each fraction were analyzed by SDS–PAGE and Western blot (Fig. 2A and B). SDS–PAGE was carried out with 12.5% (w/v) polyacrylamide gel (SuperSep™Ace, Wako, Japan). For the Western blot analysis, anti-His tag monoclonal antibody (1:1000, Qiagen, Germany) from mouse as the primary antibody and anti-mouse IgG antibody conjugated with horseradish peroxidase (1:1000, GE healthcare, UK) as the secondary antibody were used. For optimum purification of h(P)RR, 0.1% of a mild detergent, Brij35 (TaKaRa BIO, Inc., Japan), was used.

2.3. Preparation and purification of anti-(P)RR antibody

An antibody against an epitope of the 6×His-tagged human (pro) renin receptor was used. The antigen E^{221}IGKRYGEDSEQFRD^{235} (close to the NH_2-terminus of the transmembrane region of the receptor) was designed based on the sequence of the (P)RR [1,19]. This polyclonal antibody, named as anti-221/235, was produced in rabbits as it was reported previously [19]. This antibody was purified using affinity column in the buffer containing 0.5× PBS with 50% glycerol, which had the above mentioned antigen peptide as affinity ligand. The final concentration of the purified antibody was 275 μg/ml. It has been accounted that directly coupled renin on the CM5 sensor chip can bind h(P)RR at the micromolar range while prorenin can not [24] and directly coupled (P)RR on the sensor chip showed inadequate binding to renin, prorenin and the peptides [19]. As we have addressed before that this could be because of immobilization of ligands (renin/prorenin) or receptor using amine coupling that might have greatly hampered their biologically relevant conformation for binding, so we thought that their flexible conformation would have been a better choice for the kinetic analysis of ligand–receptor interaction. This notion led us to prepare the anti-221/235 antibody.

2.4. Preparation of human renin and prorenin

A cDNA coding for human prorenin (containing 383 amino acid residues from 1L-R383) with a signal sequence from sheep angiotensinogen gene at the N-terminus and a decahistidine at the C-terminus was inserted into an expression plasmid pcDNA3-sAgSP-hPren-10His [25]. The resulting plasmid was transfected into Chinese hamster ovary (CHO) cells according to Nakagawa et al. [25]. The CHO cell lines harboring human prorenin cDNA were maintained under humidified atmosphere of 5% CO_2 and 95% air in 25-cm^2 cell culture flasks (CELLSTAR, Greiner, Bio-One, Germany) till achieving 100% confluent monolayer in the DMEM medium containing 0.1 mM non-essential amino acids, 2 mM glutamine, 100 U penicillin, 100 μg streptomycin per ml and 200 nM methotrexate supplemented with 5% dialyzed fetal bovine serum. The secreted crude preparation of prorenin in the culture medium was purified as mentioned earlier [25].

The human mature renin was prepared after proteolytic cleavage of prorenin prosegment by trypsin and thus purified using Co^{2+}-chelate affinity chromatography on a TALON column and thus identified by anti-human mature renin as well as anti-pentahistidine antibodies [25]. The concentrations of renin and prorenin in the medium were measured based on the enzyme-linked immunosorbent assay according to Suzuki et al. [12]. These preparations were stored at −80 °C until further use. When the prorenin preparation was used, it was incubated at 37 °C for 1 h to avoid cryo-activation. By this treatment, prorenin showed less than 2% of its total potential activity obtained after trypsin treatment.

2.5. Binding assay of renin, prorenin and peptides to the h(P)RR using BIAcore

A novel approach for the immobilization of the (P)RR on the sensor chip through anti-(P)RR antibodies in BIAcore has recently been evaluated to investigate the interactions of renin and prorenin with the receptor [19]. Thus, real-time binding assays of human renin, prorenin and the peptides to the purified h(P)RR were performed using surface plasmon resonance technique in BIAcore 2000 machine (BIAcore AB, Uppsala, Sweden). Our previous observations demonstrated that the recombinant (P)RR when associated with anti-221/235 antibody showed the highest binding affinity for the ligands (renin, prorenin and decoy peptide; from Tables 1, 2 and 3 of [19]). Thus, considering the hypothesis of our experiment, in this study, the combination of (P)RR-anti-221/235 antibody was used to observe the kinetic analysis of the bindings of the ligands. The antibody, diluted in HBS-EP buffer at a concentration of 30 μg/ml, was allowed to immobilize on the surface of the

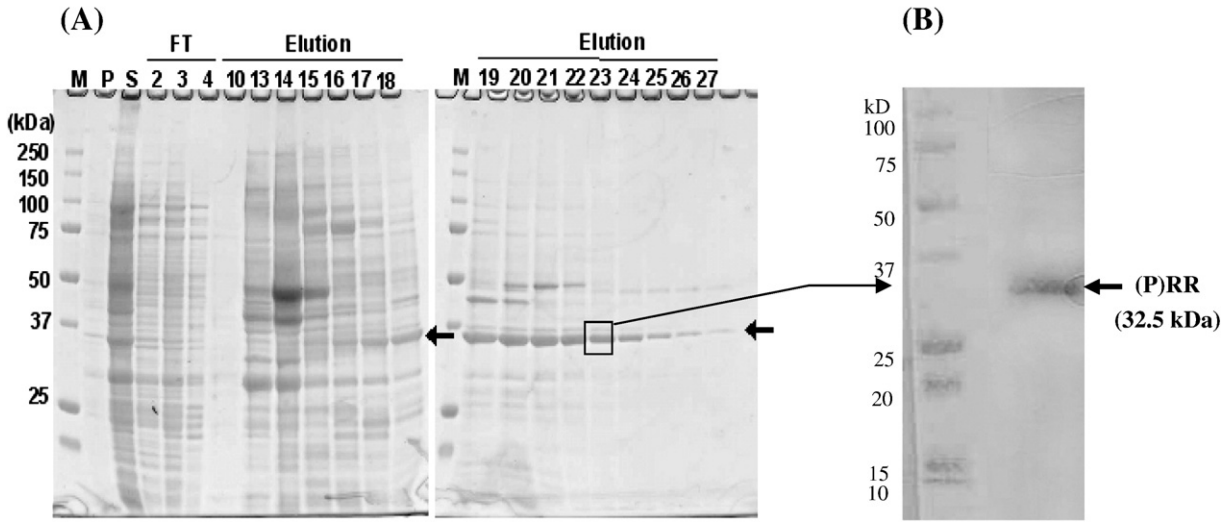


Fig. 2. SDS–PAGE and Western blot analysis of human (pro)renin receptor. The receptor was expressed in a cell free *in vitro* system using RTS 500 Wheat Germ CECF kit and purified by His Trap column chromatography. (A) SDS–PAGE was performed with 12.5% (w/v) polyacrylamide gel using SuperSep™Ace. After running on the SDS–PAGE gel, the (P)RR in the fraction number 23 was then allowed for Western blotting for further confirmation. (B) Western blot analysis of the fraction number 23 with discrete band on SDS–PAGE confirms the expression and purification of h(P)RR by Western blot using anti-His tag antibody. The fraction number 23 was used as the (P)RR preparation in this study. FT: flow through fraction.

CM5 sensor chip by amine coupling [19] and around 0.5 ng of antibodies was immobilized per square millimeter area of the surface. The resonance units (RUs) indicated the binding quantities of the analytes. The amount of immobilized (P)RR (flown at around 15 nM or 0.5 μg/ml) on the sensor chip coupled antibody was around 0.15 ng/mm^2. It was found that the receptor bound to the immobilized anti-221/235 antibody on the sensor chip in a dose-dependent manner. BIAevaluation was used to determine the association (k_a) and dissociation rate constants (k_d) of the ligands at each concentration and then their average values were used to determine the dissociation affinity constant (K_D) as the ratio of k_d/k_a. In this case, sensor chip containing immobilized anti-receptor antibody lacking the h(P)RR was used as control to determine the nonspecific bindings. A mixture of glycine (10 mM) and NaCl (150 mM) was used as the regeneration buffer (washing buffer) that helped us to avoid repeated immobilization of the antibody on the sensor chip and regeneration of the immobilized antibody on the surface of sensor chip by this buffer reproduced same binding affinity for the receptor. The resonance units (RU) that indicate the binding quantities of the renin, prorenin and the peptides are not proportional to the concentrations of the analytes but rather represent their binding mass. The HBS-EP was used as running buffer.

2.6. Binding assay of renin and prorenin to pre-adsorbed h(P)RR on the plastic surface

After dilution of the original h(P)RR preparation, 100 μl aliquots (30 nM of receptor in PBS) were allowed to immobilize on the plastic surface of 96-well plate at 4 °C for 24 h using blocking buffer (0.1% casein in phosphate-buffered saline). The K_D values for the binding of renin and prorenin to (P)RR were calculated by determining their concentration-dependent binding to the receptor. Different concentrations of renin and prorenin (0.5–3.0 nM) were incubated in 100 μl medium with pre-adsorbed (P)RR at 4 °C for 1 h. After incubation, amounts of renin and prorenin in the media were measured. Amounts of receptor-bound renin and prorenin were estimated by subtracting the remaining concentration of renin and prorenin from their initial concentration [4].

For the determination of K_m, after incubating renin and prorenin with the immobilized h(P)RR in the 96-well plate, the medium was removed and the wells were washed with ice-cold PBS. The K_m values were determined from the rate of angiotensin I production from sheep angiotensinogen at concentrations of 1.5, 1.0, 0.6 and 0.45 μM. Enzymatic activities of receptor-bound renin and prorenin were measured by angiotensin I ELISA [26] with the recombinant sheep angiotensinogen preparation [27] under standard assay condition as described previously [26].

2.7. Binding assay of renin and prorenin to h(P)RR after co-incubating with the peptides

Binding inhibition of human renin and prorenin to the h(P)RR by the peptides was performed using BIAcore and pre-adsorbed h(P)RR on the plastic surface. Human renin and prorenin (0.5 nM) were co-incubated with the fixed concentration of (80 nM) decoy and "hinge" peptides to observe the inhibition of renin and prorenin binding to the immobilized antibody-associated receptor on the CM5 sensor chip. The association (k_a) and dissociation (k_d) rate constants for the receptor–analyte interaction were determined using BIAevaluation programme as well as receptor–protein dissociation constant, K_D, was calculated as the ratio of k_d/k_a.

For equilibrium state analysis, varying concentrations of human renin or prorenin preparations (0.5–3.0 nM) in 100 μl medium and the fixed concentrations of the peptides were co-incubated with the pre-adsorbed receptors to determine the interference of the ligand receptor binding. The inhibition of the ligand binding to the pre-adsorbed receptors was calculated by using the following equation 1:

$$K_{Dapp} = K_D \times (1 + [i] / K_i) \quad (1)$$

where K_D is dissociation constant for receptor–renin or prorenin complex, K_{Dapp} is its measured value, [i] is concentration of the peptides used and K_i is dissociation constant for receptor–peptide inhibitor complex.

To confirm the intact form of renin and prorenin after binding to (P)RR, SDS–PAGE and Western blot analyses were accomplished by exploiting the opportunity of using pre-adsorbed receptors on the plastic surfaces. Renin and prorenin preparations were incubated with pre-adsorbed receptors and the reaction mixtures were removed from the surface by sample buffer. Also, after co-incubating renin/prorenin by decoy peptide with pre-adsorbed receptors, same treatment was performed. These prepared samples were then heated at 95 °C for 5 min before running them on the SDS–PAGE gel. The rabbit anti-prosegment antibody (1:1000) as the primary and the anti-rabbit IgG1 antibody conjugated with horseradish peroxidase (HRP, 1:1000) as the secondary antibody were used to detect the intact prorenin. To identify renin, human anti-renin (1:1000) and HRP conjugated anti-rabbit IgG1 (1:1000) were used as the primary and secondary antibody, respectively.

2.8. Assay of the enzyme activity of free form of mature renin as well as receptor-bound renin and activated prorenin after co-incubating with the peptides

The K_m values of the receptor-bound active renin and prorenin were calculated from the rate of angiotensin I production at 0.45, 0.6, 1.0 and 1.5 μM of sheep angiotensinogen. The two kinds of peptides, decoy and "hinge," at a concentration of 80 nM were used to investigate their effects on the activity of the free form of mature renin as well as on the receptor-bound renin and prorenin. Recombinant human renin preparation (1.0 nM) was incubated with the purified recombinant h(P)RR preparation (0.5 μg/ml) for 1 h at 4 °C. Pre-adsorbed (P)RR was also treated with renin and prorenin preparations as described in previous section. In this case, to adjust similar amounts of renin and prorenin bound to the immobilized receptor, 1.0 nM of mature renin and around 0.5 nM of prorenin were added to the immobilized receptors according to the K_D values determined in this study (Figs. 5B and 6B and Table 1). Various concentrations of sheep angiotensinogen (0.2, 0.4, 0.6, 0.8 and 1.0 μM) were incubated with mature renin, (P)RR-bound active renin and (P)RR-bound activated prorenin in the presence of either of the peptides (80 nM). The amount of angiotensin I was generated measured by Ang-I ELISA [26].

3. Results and discussion

3.1. Binding properties of renin, prorenin and peptides to h(P)RR using BIAcore

The antibody mediated immobilization of (P)RR resulted in adequate binding of human renin, prorenin and the peptides. Human (pro)renin receptor associated with immobilized anti-221/235 antibody could bind to human renin and prorenin (Fig. 4A and B). Also, bindings of R^{10P}IFLKRMPSI^{19P} (human decoy), S^{149}QGVLKEDVF^{158} (human "hinge"), L^{1P}PTD^{4P}, L^{1P}PTDTTTF^{8P}, L^{1P}PTDTTTFKRIFLKR^{15P} and A^{248}KKRLFDYVV^{257} to h(P)RR-anti-221/235 antibody complex were observed in BIAcore assay system (Fig. 3A–C). The subtracted values of the resonance units between specific and nonspecific bindings were indicated by response differences, and the figures were constructed as time vs. response difference. The data of the association (k_a) and dissociation (k_d) rate constants as well as

Table 1
Kinetic parameters of the binding of human renin, prorenin, 1^P-4^P, 1^P-8^P, 1^P-15^P, decoy, "hinge" and 248–257 peptides to the receptor associated with anti-221/235 antibody as well as enzymic activity of receptor-bound renin/prorenin and interference of their binding by the peptides.

Analytical methods	Immobilized receptors on the 96-well plates		Receptors immobilized on the CM5 sensor chip using anti-receptor antibody in BIAcore assay system								
Parameters	Renin	Prorenin	Renin[a]	Prorenin[a]	Decoy peptide[a]	"Hinge" peptide	1^P-4^P	1^P-8^P	1^P-15^P	248–257
k_a (M^{-1} s^{-1})	–	–	$2.1\times10^6\pm0.09\times10^6$	$1.8\times10^7\pm0.08\times10^7$	$1.6\times10^6\pm0.07\times10^6$	$1.6\times10^6\pm0.56\times10^6$	$1.01\times10^3\pm0.5\times10^3$	$1.4\times10^6\pm0.61\times10^6$	$1.99\times10^6\pm0.81\times10^6$	$1.0\times10^3\pm0.23\times10^3$
k_d (s^{-1})	–	–	$9.2\times10^{-3}\pm0.09\times10^{-3}$	0.02 ± 0.003	$5.6\times10^{-3}\pm1.0\times10^{-3}$	0.03 ± 0.002	0.0321 ± 0.001	0.0709 ± 0.012	0.015 ± 0.002	0.0407 ± 0.012
K_D (nM)	4.5 ± 1.5	1.0 ± 0.1	4.4 ± 1.8	1.2 ± 0.4	3.5 ± 1.6	17.0 ± 2.6	$3.19\times10^4\pm1.1\times10^4$	52 ± 12.1	7.56 ± 3.7	$4.07\times10^4\pm1.7\times10^4$
K_i of decoy peptide (nM)	16.7 ± 3.5	15.1 ± 2.8	–	–	–	–	–	–	–	–
K_i of hinge peptide (nM)	37.1 ± 6.2	30.7 ± 3.1	–	–	–	–	–	–	–	–
K_m (μM)	0.1 ± 0.02	0.1 ± 0.04	–	–	–	–	–	–	–	–

[a] From our previous study [19].

dissociation constants (K_D) for the interaction of renin, prorenin and each peptide with the immobilized (P)RR were determined using Langmuir 1:1 kinetic binding model as described previously [19] and summarized in Table 1. The bindings of $L^{1P}PTD^{4P}$ and $A^{248}KKRLFDYVV^{257}$ peptides to the (P)RR-anti-His tag antibody complex were investigated earlier [19], and in this study, these were used as reference peptides to compare lower bindings.

As reported in our previous work [19], this study confirmed the similar association (k_a) and dissociation (k_d) rate constants of renin and prorenin for immobilized (P)RR ($2.1\times10^6\pm0.09\times10^6$ and $1.8\times10^7\pm0.08\times10^7$ M^{-1} s^{-1} as well as $9.2\times10^{-3}\pm0.09\times10^{-3}$ and 0.02 ± 0.003 s^{-1}, respectively), while the values of K_D were 4.4 and 1.2 nM, respectively. These data showed that although the binding affinities for human prorenin and renin to the immobilized receptors were within the nanomolar range, prorenin had almost four times higher affinity than that of human mature renin. More detailed kinetic analysis demonstrated that prorenin had a higher association rate ($k_a=1.8\times10^7$ M^{-1} s^{-1}) compared to that of renin molecule ($k_a=2.1\times10^6$ M^{-1} s^{-1}). To elucidate the reason for such differences in binding affinities and to determine a key recognition feature, we tested the binding properties of discrete peptides from the N-terminal side of prorenin prosegment such as 1^P-8^P and 1^P-15^P along with 1^P-4^P and 10^P-19^P [19] to the immobilized (P)RR. Their kinetic analyses revealed that both R^{10P}IFLKRMPSI^{19P} and L^{1P}PTDTTTFKRIFLKR^{15P} peptides showed more stable complex with (P)RR as reflected from their K_Ds (3.5 and 7.6 nM, respectively) compare to other peptides (Table 1). This is due to the presence of a common sequence I^{11P}FLKR^{15P} called "handle" region peptide, which has been reported as a crucial region in prorenin prosegment for its interaction with other proteins by Suzuki et al. [12]. Thus, the co-injection of decoy peptide with renin and prorenin not only reduced the resonance unit for prorenin but also for renin binding to h(P)RR (Fig. 4A and B). As binding of the peptides to (P)RR were directly observed in BIAcore and binding amounts depend on the molecular mass of ligands, so reduction in resonance unit indicated inhibition of renin/prorenin binding by the peptides. The reason for reduction of prorenin binding to (P)RR by decoy is understandable whereas reduction of renin binding, which lacks the prosegment sequence, by this peptide is unclear. Augmented or decreased biological activities of the receptors and/or ligands due to their interaction via change in their conformation have been discussed in many studies. For example, T cell receptor engagement by cognate peptide–MHC ligands induces a conformational change in the CD3 complex of thymocytes that involves in thymic selection [28]; the host cell receptor for subgroup A avian leukosis and sarcoma viruses binds specifically to the subgroup A envelope glycoprotein (Env-A) and causes conformational changes in Env-A that correlate with its conversion from a fusion-inactive to a fusion-active state [29]; direct binding of α5β1 to urokinase-type plasminogen activator receptors (uPARs) changes the conformation and matrix-binding properties of α5β1 and functions of this complex could be blocked by short β1-chain peptides without affecting α5β1-mediated fibronectin binding itself point to important conformational differences between free and uPAR-bound α5β1 [30]. Taking these facts into account the putative reason for the inhibition of renin binding to (P)RR by the decoy peptide could be due to the conformational change in (P)RR mediated by this peptide which has ultimately been reflected in the reduction of resonance units for renin binding after attenuating its affinity (as shown in Fig. 9). This also arises a possibility of having either multiple or common recognition sites or both in renin and prorenin through which they can communicate with the receptor. Several *in vitro* and *in vivo* studies have confirmed that after binding to (P)RR, renin and prorenin perform their catalytic activity [1–4,31–33], thus indicating that their binding must involve sequences other than the active sites. These facts together have led us to hypothesize that a peptide sequence common to both of these molecules might have a role in the binding.

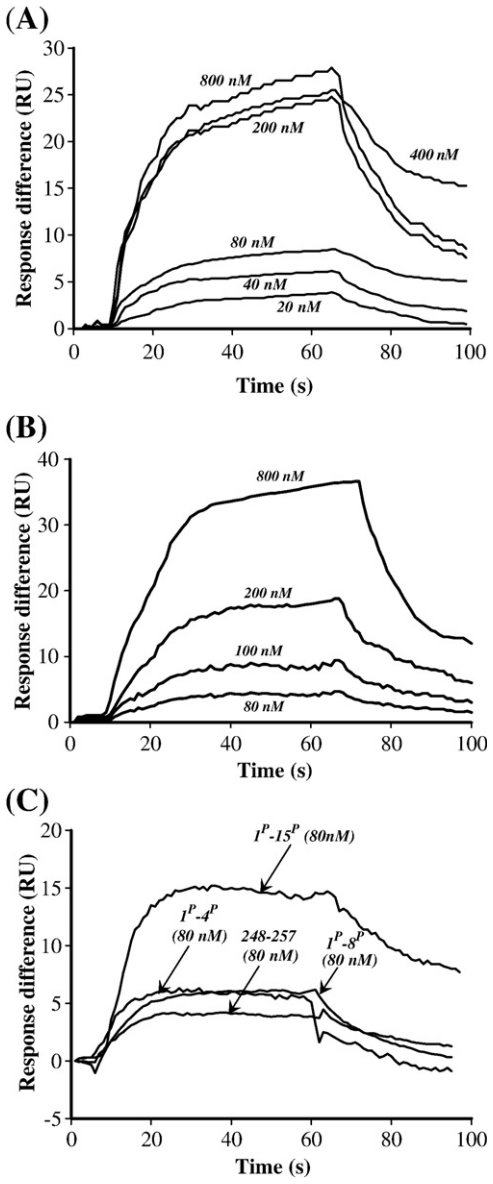


Fig. 3. Concentration dependent binding of peptides to (P)RR. The decoy [R^{10P}IFLKRMP-SI^{19P} in (A)] and "hinge" peptides [S^{149}QGVLKEDVF^{158} in (B)] to h(P)RR associated with anti-h(P)RR antibody immobilized on the surface of CM5 sensor chip. Different concentrations of decoy (20, 40, 80, 100 and 800 nM) and "hinge" (80, 100, 200 and 800 nM) peptides were allowed to bind to h(P)RR. In (C), peptide 1^P-15^P including the sequence of HRP showed the highest response of resonance units while the others gave a little response. Sensor chip containing immobilized anti-h(P)RR antibody but lacking the h(P)RR was used as control to determine nonspecific binding of the peptides. Response differences were calculated by subtracting the response signal of nonspecific binding from that of specific binding.

Based on this hypothesis a peptide termed as "hinge" from the flexible region of their N- and C-domain was designed from the tertiary structure of renin as well as predicted model of prorenin molecule (as shown in Fig. 1). Its binding properties were determined and compared with the peptide A^{248}KKRLFDYVV^{257}. Among these two, the "hinge" peptide showed higher affinity for the receptor with a K_D of 17.0 nM calculated from its k_a and k_d values. Further, co-incubation of renin and prorenin with "hinge" revealed reduction in resonance signal for the binding of these molecules to the immobilized receptor. This supports our hypothesis regarding the multiple binding regions of prorenin. Further, the data from the kinetic analysis indicate that decoy peptide has higher affinity to (P)RR than "hinge" peptide. As the peptide sequences of both decoy and "hinge" are conserved in prorenin, so that explains the reason behind higher affinity of prorenin for the receptor compared to that of renin.

3.2. Binding properties of renin and prorenin to pre-adsorbed h(P)RR

Figs. 5A and 6A show saturation of binding of renin and prorenin to the immobilized h(P)RR on the surface of the plastic wells, respectively. Amounts of bound renin and prorenin were determined by subtracting the remaining concentration of unbound renin and prorenin, respectively, from their initial concentration. Corrections were made for nonspecific binding to control well which did not have (P)RR. The K_D values of renin and prorenin to h(P)RR were estimated 4.5 and 1.0 nM, respectively (Figs. 5B and 6B). Bindings of renin and prorenin to (P)RR were inhibited while co-incubated with decoy or "hinge" peptides at a concentration of 80 nM. The K_i values of the peptides were determined according to Eq. 1 (Figs. 5B and 6B). These data are summarized in Table 1. The K_i values were estimated to be 16.7 and 37.1 nM, respectively by the decoy and "hinge" region peptides for the inhibition of renin binding, whereas for prorenin, these values were measured at 15.1 and 30.7 nM, respectively. Due to higher dissociation constants, the co-incubation of renin and prorenin with the peptides such as $L^{1P}PTD^{4P}$, $L^{1P}PTDTTTF^{8P}$ and A^{248}KKRLFDYVV^{257} were effective neither in BIAcore studies nor in equilibrium state on the plastic surface.

Use of purified (P)RR made it possible to immobilize the receptor to the plastic surface of 96-well plates. It provided a stable form of (P)RR in a functionally reproducible state for ligand binding to the receptor as shown in Figs. 5 and 6. The purified h(P)RR was confirmed by Western blotting (Fig. 2) whereas the immobilized h(P)RR on the plastic surfaces was demonstrated by the anti-(P)RR antibody. The prorenin bound to the pre-adsorbed receptor was observed according

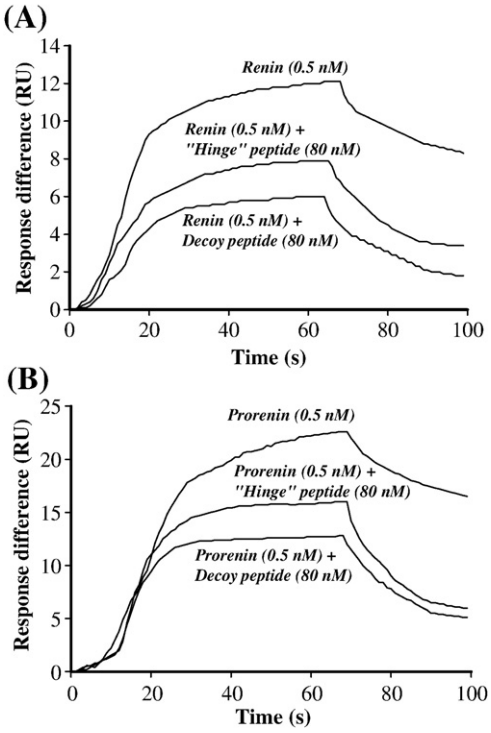


Fig. 4. Human (pro)renin receptor binding to human renin and prorenin. Recombinant human renin (A) and prorenin (B) at 0.5 nM bound to h(P)RR associated with anti-h(P)RR antibody on the surface of CM5 sensor chip using the surface plasmon resonance technique. Sensor chip containing immobilized anti-h(P)RR antibody lacking the h(P)RR was used as control to determine nonspecific bindings. Response differences were calculated by subtracting the response signal of nonspecific binding from that of specific binding. Decreased response unit after addition of decoy and "hinge" peptides (80 nM) indicated interference of the binding of human renin and prorenin by these peptides.

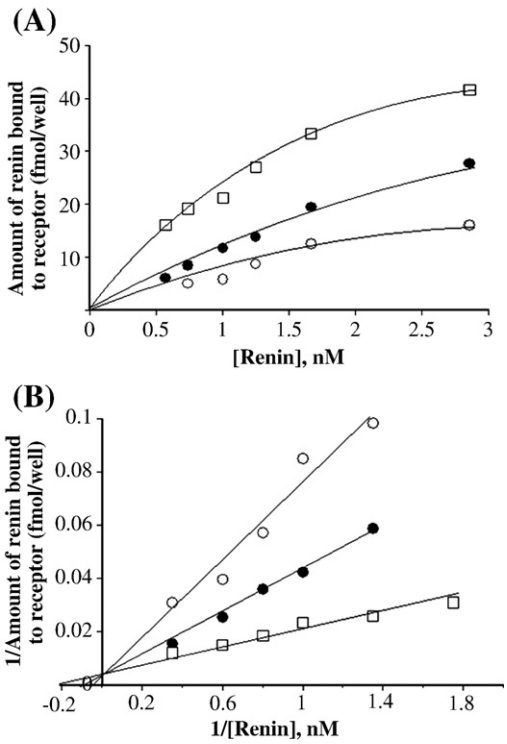


Fig. 5. Saturation of the binding of human renin to h(P)RR (A) pre-adsorbed on the plastic surface, determination of dissociation constant (K_D) of renin for (P)RR and K_i for decoy as well as "hinge" peptide (B). In (A), different concentrations of renin without peptide (open squares), with "hinge" peptide (closed circles) and with decoy peptide (open circles) were allowed to bind with the recombinant immobilized receptors. Amount of bound renin was determined by subtracting the remaining amount of unbound renin from its initial amount. (B) Double reciprocal plots for the determination of K_D of renin and K_i of the peptides. The K_D value of human renin was calculated as 4.5 nM. The K_i values were determined using the following equation: $K_{Dapp} = K_D \times (1 + [i]/K_i)$, where K_{Dapp} values for renin were estimated to be 25.0 and 14.25 nM when co-incubated with decoy and "hinge" peptides, respectively.

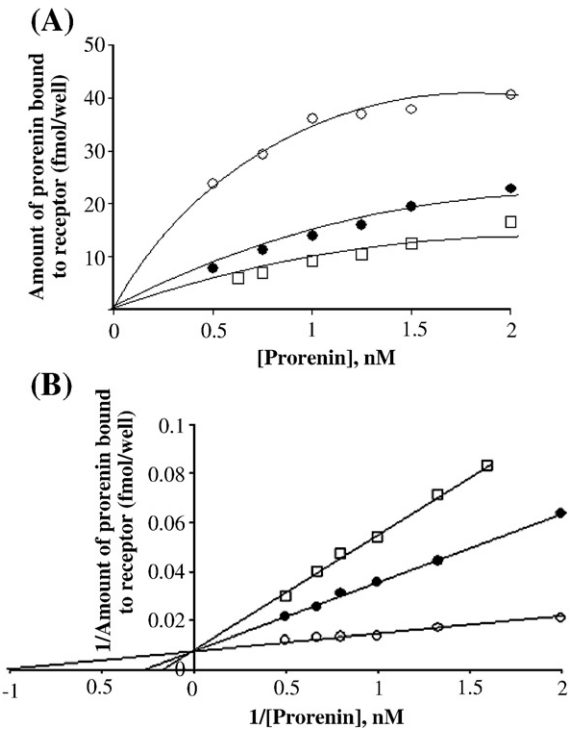


Fig. 6. Saturation of the binding of human prorenin to h(P)RR (A) pre-adsorbed on the plastic surface, determination of dissociation constant (K_D) of prorenin for (P)RR and K_i for decoy as well as "hinge" peptide (B). In (A), different concentrations of prorenin without peptide (open circles), with "hinge" peptide (closed circles) and with decoy peptide (open squares) were allowed to bind with the recombinant immobilized receptors. Amount of bound prorenin was determined by subtracting the remaining amount of unbound prorenin from its initial amount. (B) Double reciprocal plots for the determination of K_D of prorenin and K_i of the peptides. Results showed that the K_D value of human prorenin was 1.0 nM. The values were determined using the following equation: $K_{Dapp} = K_D \times (1 + [i]/K_i)$, where K_{Dapp} values for prorenin were estimated to be 6.3 and 3.6 nM when co-incubated with decoy and "hinge" peptides, respectively.

to the method described in our previous study using an antibody against the prosegment sequence of prorenin [2] and human renin bound to the pre-adsorbed receptors on the plastic surfaces was also identified by anti-human renin antibody. It is already established that receptor-bound prorenin showed catalytic activity that is comparable to that of mature renin either bound to the receptor or in the soluble form [1–4,31–33]. An *in vivo* experiment with double transgenic mice generating human angiotensinogen with noncleavable human prorenin showed possibly elevated Ang-I producing capability [34]. In this *in vitro* study, using Western blot analysis, it was confirmed that even after binding to (P)RR prorenin keeps its prosegment as its intact form (Fig. 7, lane 3).

Recognition of receptor-bound prorenin using an antibody against the epitope of C-terminal region of prorenin prosegment clearly indicates that at least this region is not responsible for binding. Also, we previously reported that a peptide sequence from the C-terminal part of the prorenin prosegment, 30^P-36^P, showed no response against the prorenin binding to (P)RR expressed on COS-7 cells [2]. On the other hand, decoy peptide including HRP from the N-terminus of prorenin prosegment competitively inhibited the binding of renin and prorenin to (P)RR (Figs. 5B and 6B). Western blot analysis documented the lower binding of prorenin to (P)RR while co-incubated with decoy (Fig. 7, lane 4). Although some studies have reported that the decoy peptide including HRP did not have any effect on the action of prorenin to (P)RR [31–33], present *in vitro* studies using kinetic (BIAcore assay system) and equilibrium (adsorbed on plastic surfaces) states as well as the Western blot data revealed that this

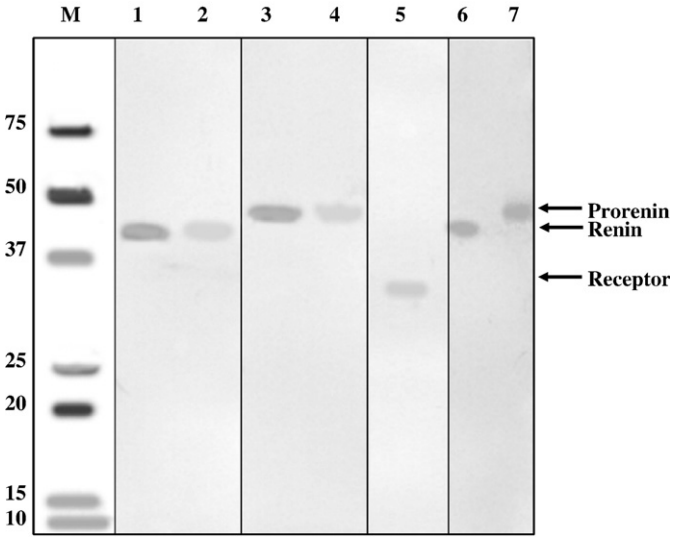


Fig. 7. Western blot analysis of the mature renin and intact prorenin molecules after binding to h(P)RR. Pre-adsorbed h(P)RR was incubated with renin and prorenin. Lanes 1 and 3 show the intact form of renin and prorenin, respectively, after binding to (P)RR. When decoy peptide was co-incubated with renin and prorenin, the intensity of the bands in lanes 2 and 4 compare to those in lanes 1 and 3 indicates their lower binding to (P)RR. Lanes 6 and 7 were developed using renin and prorenin in the medium while lane 5 designates the band of (P)RR.

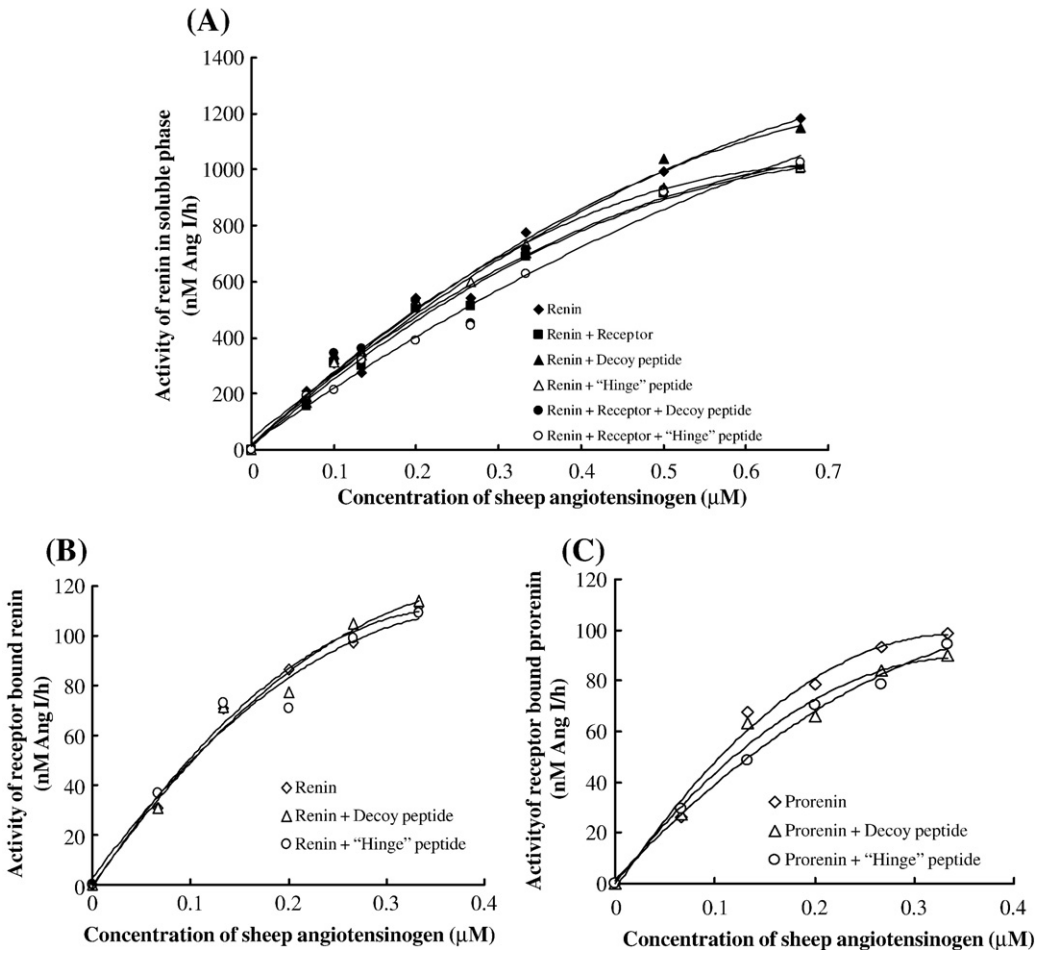


Fig. 8. Effect of decoy and "hinge" peptides on the activity of free form of mature renin (A), receptor-bound renin (B) and receptor-bound activated prorenin (C). Free form of mature renin was incubated under different conditions. Human renin and prorenin (0.5 nM) preparation was incubated with the immobilized receptors at 4 °C for 1 h and then activities of renin and activated prorenin were measured by standard assay conditions using peptides at 80 nM.

peptide has a role in prorenin binding to h(P)RR. Therefore, such reactivity of (P)RR on the membrane is probably sensitive under the circumstances on and/or around the membranes. We were able to use the same system to show interference of renin and prorenin binding by "hinge" peptides. These findings of competition between renin/prorenin and the peptides for the binding sites in (P)RR explain the reason for reduction in resonance units of their binding when co-incubated with the peptides in BIAcore assay system. This assay system will, therefore, be utilized as an effective tool for (P)RR related studies in the equilibrium state.

3.3. Effect of the peptides on the activity of free form of mature renin, renin bound to (P)RR and the bound form of activated prorenin

The free form of mature renin, receptor-bound renin and activated prorenin bound to receptor had similar K_m values for sheep angiotensinogen estimated at around 0.1 μM. Many studies have already showed that the decoy peptide inhibited not only the non-proteolytic activation of prorenin by inhibiting the binding of prorenin to the (P)RR [2,14–16,19] but also renin binding to the receptor [19]. Whether the decoy and "hinge" peptides can inhibit the enzymic activities of receptor-bound renin and activated prorenin were also investigated in this study. After allowing renin and prorenin to bind with the pre-adsorbed receptors, there enzymic activities were measured using the substrate sheep angiotensinogen in the presence of the peptides (80 nM). The two peptides did not inhibit the activity of mature renin and bound form of renin as well as prorenin under the assay condition. Fig. 8A–C shows the effects of both peptides on the activity of free form of mature renin as well as receptor-bound activated renin and prorenin. Further, Cumin et al. reported earlier that human renin activity could be inhibited by the synthetic peptides derived from the prosegment sequence of prorenin, where they used micromolar ranges of peptides [35]. But in this study using nanomolar concentration, these peptides showed no effect on the catalytic activity of the free form of mature renin, or on the receptor-bound activated renin as well as prorenin (Fig. 8A–C), which indicated that at least at these concentrations, peptides did not have any effect on the interaction with renin substrate, angiotensinogen. Therefore, as outlined in Fig. 9, these peptides only interfered with the bindings of renin and prorenin to h(P)RR under the present assay condition.

The importance of this *in vitro* study could also be correlated with the physiological system as well. The existence of soluble form of (P)RR has recently been reported [36]. The plasma prorenin concentration is 10 times higher than that of mature renin [12]. Actually, the concentration of renin in plasma is in the picomolar range, but in the tissue fluid, it is almost 100 times higher. Even though the binding percentage is very low, activation of angiotensinogen or second messenger pathways by receptor-bound renin or prorenin could contribute in the pathophysiology of the circulating and/or local renin angiotensin system.

4. Conclusions

In conclusion, the use of purified anti-(P)RR antibody (anti-221/235 antibody) allowed adequate immobilization of (P)RR on sensor

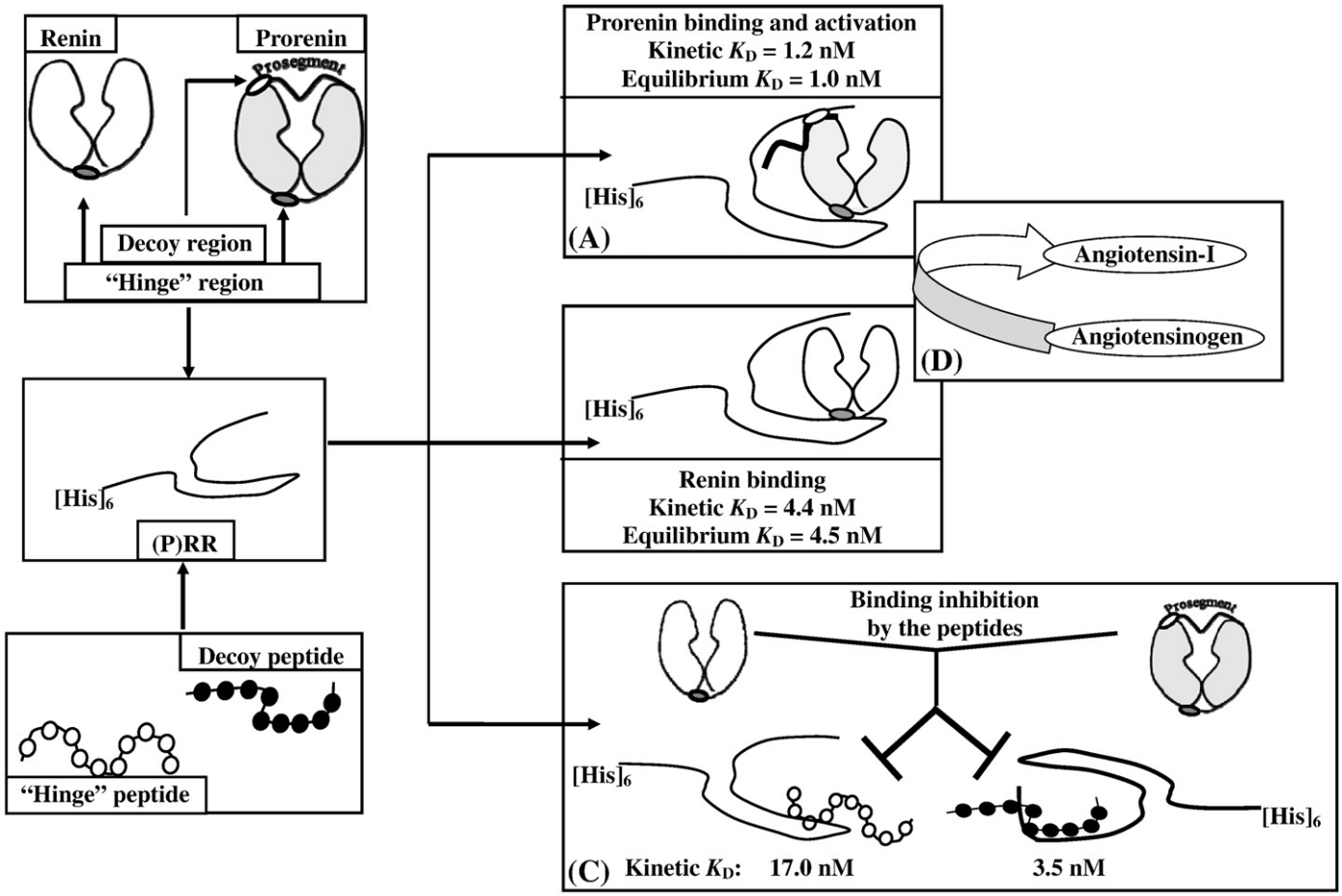


Fig. 9. Possible binding mechanism of human renin and prorenin to (pro)renin receptor. This diagram shows the models of renin, prorenin (with the decoy and "hinge" binding sites), h(P)RR containing 6×His tag at the C-terminal instead of the transmembrane part, the decoy and "hinge" peptides. (A) Prorenin presumably binds to the (P)RR through its decoy sequence in prosegment and "hinge" region. (B) Renin binds to the receptor via the "hinge" region present between the N- and C-domain. (C) Direct binding of decoy and "hinge" peptides reduced the resonance signal for renin and prorenin binding to the receptor when co-incubated with each other. (D) Decoy and "hinge" peptides interfered with the renin and prorenin binding to the receptor but did not inhibit renin activity.

chip for the binding assays of renin, prorenin and the peptides. This study showed that decoy peptide had higher binding affinity ($1/K_D$) than the "hinge" peptide and the decoy is only conserved in prorenin molecule whereas the "hinge" is present both in mature renin and prorenin molecules. These *in vitro* findings, thus, revealed that the prorenin molecule has two high affinity sites and renin has a single site through which they can interact with (P)RR.

Acknowledgements

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