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Acid-activated prorenin binds to (pro)renin receptor *in vitro*

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ABSTRACT

Binding properties of acid-activated prorenin to (pro)renin receptor [(P)RR] was investigated *in vitro* to discuss possible roles of such reversibly acid-activated prorenin in the renin angiotensin (RA) system. Prorenin was acidified at pH 3.3, 4.5, 5.5, 6.5, and its activation level was measured at 1, 2, 4, 8, 12, and 25 h. Prorenin, activated non-proteolytically in time- and pH-dependent manners, was verified by Western blot analyses. Acidification of prorenin for 25 h at pH 3.3, 4.5, 5.5, and 6.5 showed 78%, 54%, 34%, and 20% activities, respectively when compared with the renin activity of trypsinized prorenin as 100%. Additionally, the binding properties of acidified prorenin to (P)RR were elucidated both at the equilibrium state and in the kinetic state using BIAcore. BIAcore assay showed that acidified prorenin at pH 3.3, 4.5, 5.5, and 6.5 had apparent K_D of 1.57×10^4, 14.1, 8.29, and 8.04 nM, respectively while native prorenin at pH 7.4 had a K_D of 7.8 nM. At equilibrium state, K_D of native prorenin was 1.42 nM whereas apparent K_D varied from 1.25 to 5.0 nM for the prorenin acidified at pH 4.5, 5.5, and 6.5. The K_m values of free forms of acidified prorenin at different pH (0.33–0.5 μM) was almost similar to those of (P)RR-bound forms of acidified prorenin (0.5–0.77 μM). These *in vitro* data indicate that prorenin acidified *in vivo* possibly modulate RA system in receptor-dependent and/or -independent manners which could ultimately lead to the pathogenesis of diseases.

1. Introduction

Prorenin, having a prosegment part of 43 amino acid residues attached to the N-terminus of mature renin, plays an important role in the renin angiotensin (RA) system. Activation of prorenin takes place *in vitro* either proteolytically or non-proteolytically. Proteolytic activation occurs by the effect of some endopeptidases such as trypsin or cathepsin B [1,2], thereby causes irreversible removal of the prosegment. Non-proteolytic activation has been reported to occur under acidic pH (known as acid activation) and/or low temperature (called cryo-activation) [3,4], which reversibly unfolds the prosegment of prorenin through conformational change. Prorenin can also be activated non-proteolytically by protein–protein interactions e.g., receptor–ligand and antibody–ligand interactions [5–7]. Kinetic studies of the non-proteolytic activation process have indicated that an equilibrium exists between the closed (inactive) and open (active) forms of prorenin [3,7,8].

Activation of prorenin in tissues by (pro)renin receptor [(P)RR] [9] plays an important role in the pathophysiology of many end-stage organ damage related disorders. (P)RR-bound prorenin, through either angiotensin II-dependent or -independent or both pathways, contributes to the development and progression of diabetic nephropathy and retinopathy, cardiac and glomerular fibrosis [10–12], and hypertension and heart failure [13]. Prorenin activation by (P)RR has also been reported to likely be associated with brain development [14] and neuronal cell differentiation [15].

Under normal physiological condition, prorenin circulates in human plasma in excess of renin (usually 10 times higher), but its concentration may increase by around 100 times in pathophysiological conditions of diseases like diabetes [16]. Even in certain body fluids, such as ovarian follicular fluid and amniotic fluid, prorenin concentrations may approach 100 times the levels found in plasma [2]. Although activated prorenin in circulating plasma is around 2% of the total prorenin [3] but in case of pathological conditions, the cumulative percentage of circulating activated prorenin would increase. This could further deteriorate the condition by activating not only local RA system but also circulating RA system with the help of recently reported truncated form of soluble (P)RR [17].

On the other hand, some pathological conditions e.g., metabolic acidosis, inflammations, mast cells degranulation, and lysosomes degradation, etc., could change local pH [18] that might lead to increase the level of active prorenin, through which (P)RR may

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modulate local RA system. A recent study has reported that metabolic acidosis induces proteinuria by increasing the expression of RA system components in rats without any change in transforming growth factor-β and nuclear factor-kB [19]. Moreover, vacular H^+-ATPases, the proteins that associate (P)RR on the cell membrane, have been reported to play important roles in the acidification of intracellular compartments and cellular pH homeostasis by releasing H^+ regionally which could activate prorenin [20].

Prorenin activated non-proteolytically could, therefore, possibly modulate circulating or local RA system more potently by generating Ang-I. The biochemical properties of renin, prorenin, decoy peptide containing the handle region sequence, and hinge region peptide for binding to (P)RR have been elucidated using different experimental approaches [5,6,9,21,22]. But those properties of acid-activated prorenin for binding to (P)RR are yet to be investigated. In this paper, the binding properties of acidified prorenin preparations to recombinant (P)RR has been elucidated *in vitro*.

2. Materials and methods

2.1. Preparation of recombinant human prorenin and (pro)renin receptor [(P)RR]

Recombinant human prorenin was prepared using Chinese hamster ovary (CHO) cells according to the method described by Nakagawa et al. [23]. The recombinant human (P)RR (32.5 kDa) was *in vitro* expressed and purified according to the method of Nabi et al. [21].

2.2. Acidification of prorenin by dialysis

Prorenin preparation was acidified by the method of dialysis against a buffer solution containing 5 mM EDTA and 1 mg/ml bovine serum albumin (BSA). The BSA preparation was thermally treated at 60 °C for 4 h to inactivate contaminated proteases that might be present within BSA preparation. The pH of BSA/EDTA buffer was adjusted to 3.3, 4.5, 5.5, and 6.5 by using 6 M HCl or 5 M NaOH. For the acidification, prorenin preparation at a volume of 250 μl and at a concentration of 40 nM was inserted into each of 4 Dialysis Cassettes [Slide-A-Lyzer 10K, Thermo (PIERCE)]. These Cassettes were then placed separately in 4 beakers containing 500 ml of BSA/EDTA buffer having 4 different pH (3.3, 4.5, 5.5, and 6.5), and incubated at 4 °C for 25 h with mild stirring. During dialysis, acidified prorenin preparations were collected from each of the Dialysis Cassette at different time intervals (1, 2, 4, 8, 12, and 25 h) to observe their time- and pH- dependent activations. Acidified prorenin preparations, which were dialyzed for 25 h only, were used for Western blot analysis, K_m determination, reinactivation experiment, and above all for binding experiment. It is important to note here that all dialyzed acidified prorenin preparations were treated with buffer containing neutral pH of 7.4 immediately before commencing any sort of experiment. Dilution factor of prorenin during dialysis was taken into account when we determined the final concentration of prorenin.

2.3. SDS–PAGE and Western blot analysis of acidified prorenin

SDS–PAGE of acidified prorenin as well as native prorenin was carried out with 12.5% (w/v) polyacrylamide gel (SuperSep™ Ace, Wako, Japan) followed by Western blot analysis. Western blot was performed using two types of primary antibodies named anti-prosegment antibody (1:1000 diluted in TBS) and anti-renin antibody (1:1000 diluted in TBS). Against these primary antibodies, anti-goat IgG antibody conjugated with horseradish peroxidase (1:1000 diluted in TBS) was used as secondary antibody.

2.4. Measurement of the renin activity of acidified prorenin

For the determination of renin activity, the concentration of acidified prorenin at each pH (for both activation and re-inactivation experiments) was used 1.3 nM. The activities of acidified prorenin were measured at 37 °C by Ang-I ELISA [24] with the recombinant sheep angiotensinogen [25] under standard assay condition [24]. To compare the activity of acid-activated prorenin with that of native prorenin (1.3 nM), latter one was treated with 1 mg/ml of trypsin for different time intervals (0, 5, 15, 30, 45, and 60 min) at 25 °C, and then activity of the trypsinized prorenin was measured using Ang-I ELISA [24]. Moreover, activities of the acid-activated prorenin (acidified for 25 h) were measured using different concentrations of sheep angiotensinogen, and from these activities double reciprocal plot was constructed to determine the K_m values of the activated prorenin in the soluble phase. Furthermore, immediate after the completion of dialysis for 25 h, prorenin preparation was neutralized at pH 7.4 using PBS to observe the reinactivation of prorenin. After starting of the neutralization process at around neutral pH, activity of acidified prorenin was measured at different time intervals (1, 2, 4, 8, 12, and 25 h) at 37 °C.

2.5. Real-time binding of acid-activated prorenin to recombinant human (P)RR

Real-time binding of acid-activated prorenin was observed using surface plasmon resonance technique in BIAcore assay system (Uppsala, Sweden). In this study, an anti-His-tag-(P)RR antibody (QIAGEN, GmbH, Germany) was immobilized on the CM5 sensor chip through amine coupling using N-ethyl-N1-dimethylaminopropyl carbodiimide (EDC) and N-hydroxysuccinimide (NHS) according to our previous protocol [21,22]. The (P)RR at a concentration of 30 nM was injected (10 μl/min) and allowed to associate with the anti-(P)RR antibody through the six histidine residues tagged at the C-terminus of (P)RR. Then, different concentration of prorenin was injected (flow rate: 10 μl/min) to bind to the immobilized receptor associated with the antibody. This binding of prorenin to (P)RR was expressed in terms of resonance unit. The flow cells, which were activated with EDC/NHS, treated with only HBS (HEPES buffered saline) and finally, blocked by ethanolamine, were used as control cells. Any binding response observed in these cells was considered as non-specific binding and finally, specific binding was obtained after subtracting non-specific binding from the total binding. The surface was regenerated by injecting a mixture of glycine (10 mM) and NaCl (150 mM) at pH 2.0 to avoid repeated coupling of anti-(P)RR-antibody. The association (k_a) and dissociation (k_d) rate constants for the binding of prorenin to the receptor were measured, and the dissociation constants (K_D) were determined using Langmuir 1:1 kinetic binding model.

2.6. Binding of acid-activated prorenin to h(P)RR pre-adsorbed on plastic wells

Binding experiment of prorenin to the pre-adsorbed receptors at equilibrium state was performed with few modifications as described previously [5,21,26]. Briefly, 200 μl of (P)RR (30 nM) was 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). Each well with pre-adsorbed (P)RR was washed with ice-cold PBS before incubating with 200 μl of different concentrations of prorenin (0.16–5.0 nM) at 4 °C for 1 h. The K_D values for the binding of prorenin to (P)RR were calculated by determining their concentration-dependent binding to the receptor [6].

For the determination of K_m, after incubating prorenin (1.3 nM) with the immobilized (P)RR in the wells of 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, 1.25, 1.0, 0.8, 0.6, 0.45, 0.30, and 0.15 μM.

3. Results

3.1. Western blot analysis

Using anti-prosegment antibody and anti-renin antibody, Western blot analysis showed that all acidified prorenin preparations (treated at pH 3.3, 4.5, 5.5, and 6.5) traveled similar distances as the control i.e., native prorenin at pH 7.4 (Fig. 1A and B). On the other hand, the stained bands after trypsinization of prorenin preparation were dominantly observed at migration distances as same as those of mature renin on the Western blot analysis using anti-renin antibodies (Fig. 1B).

3.2. Non-proteolytic activation of prorenin by acid treatment

When native prorenin was treated with trypsin at 25 °C at different time intervals, the highest activity (26.5 ng Ang-I/ml/h) was observed at 5 min. But, the trypsin treatment for more than 15 min, the renin activity was observed at much reduced level. On the other hand, acid-activation levels of prorenin had reached plateaus within 25 h for each acidified conditions. Prorenin incubated at pH 3.3 generated higher renin activity (20.7 ng Ang-I/ml/h) compared to those treated at pH 4.5, 5.5, and 6.5, respectively (14.2, 11.5, and 6.2 ng Ang-I/ml/h, respectively). When compared with the activity of trypsin-treated prorenin (26.5 ng Ang-I/ml/h that was considered as 100% activation level of prorenin), acid-activated prorenin showed 78, 54, 34, and 20% activity after acidification for 25 h at pH 3.3, 4.5, 5.5, and 6.5, respectively. Time-dependent activation curve of prorenin was also observed when it was non-proteolytically activated at pH 3.3, 4.5, 5.5, and 6.5 for several incubation times (1, 2, 4, 12, and 25 h; Fig. 1C). At every time point of acidification, the renin activity of prorenin treated at lower pH was higher than that treated at higher pH. Cryo-activation of native prorenin was measured approximately 2%, but this activity was maintained at a minimum level of 0.6% by treating the prorenin preparation at 37 °C for 1 h. Re-inactivation of prorenin preparations at different pH was also observed by incubating them at pH 7.4 and 37 °C for different incubation times (Fig. 1D). The rate of re-inactivation was higher for prorenin at pH 3.3, and within 12 h of incubation the activity of prorenin at all pH reduced to almost the same point-activity (~10%). The K_m of acidified prorenin treated at pH 3.3, 4.5, 5.5, and 6.5 varied from 0.33 to 0.50 μM.

3.3. Real-time binding kinetics of acidified prorenin to the immobilized h(P)RR

Trypsin treatment of prorenin preparations revealed that acidified prorenin showed different levels of activation at different pH values, and some percentages of prorenin still remained as inactive and intact forms. As the activation rate and plateau level of prorenin varied with pH due to acidification (Fig. 1), the dissociation constants (K_D) for the binding of acidified prorenin to the immobilized (P)RR were calculated as apparent values. BIAcore assay showed the dose-dependent binding of prorenin at every pH (Fig. 2), and their association (k_a) and dissociation rate (k_d) constants as well as K_D values were summarized in Table 1. The K_D of prorenin treated at pH 3.3 was almost 10,000 times higher than that treated at other pH.

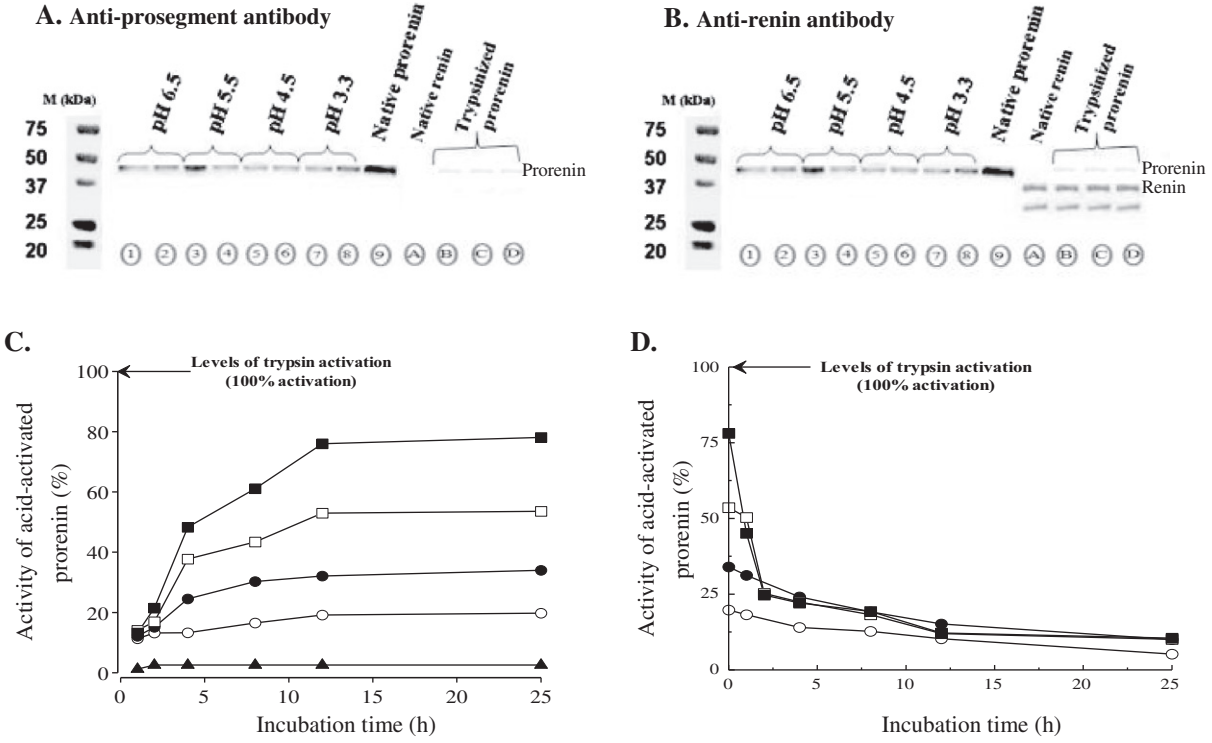


Fig. 1. Western blot analysis and, activation and re-inactivation experiments of acidified prorenin treated at several acidic pHs. (A) Western blot analysis of acidified prorenin using anti-prosegment antibody. (B) Western blot analysis of acidified prorenin using anti-renin antibody. Lanes in the figures (both A and B) indicate the following states of (pro)renin. Lanes 1, 3, 5, and 7: acidified prorenin immediately after completing dialysis at pH 6.5, 5.5, 4.5, and 3.3, respectively before neutralization using PBS (pH 7.4); lanes 2, 4, 6, and 8: acidified prorenin 1 h after neutralizing with PBS (pH 7.4) on the completion of dialysis at pH 6.5, 5.5, 4.5, and 3.3, respectively; lane 9: native recombinant human prorenin; Lane A: Native recombinant human renin; lanes B–D: recombinant prorenin treated with trypsin (1 mg/ml) at 25 °C for 5, 15, and 25 min, respectively. (C) Percent activity of acidified prorenin at different pH for different time intervals. pH 3.3: closed squares; pH 4.5: open squares; pH 5.5: closed circles; pH 6.5: open circles; and control (native prorenin at pH 7.4): closed triangles. (D) Rates of re-inactivation of acidified prorenin at different pH for different time intervals. pH 3.3: closed squares; pH 4.5: open squares; pH 5.5: closed circles; and pH 6.5: open circles.

3.4. Binding kinetics of acidified prorenin at equilibrium state

Since BIAcore binding assay revealed that prorenin treated at pH 3.3 had very high K_D value compared to prorenin acidified at pH 4.5, 5.5, and 6.5, binding of prorenin treated at pH 3.3 were not tested in the case of pre-adsorbed (P)RR. Moreover, of the total prorenin, 20–50% became activated after acidification at pH 4.5, 5.5, and 6.5, and rest of them either was partially activated or held their native forms. As a result, K_D values of the acidified prorenin were considered as apparent. The apparent K_D values of active prorenin treated at pH 4.5, 5.5, and 6.5 for their bindings to the immobilized h(P)RR were measured at 5.0, 2.0, and 1.25 nM, respectively (Table 1).

3.5. Renin activities of receptor-bound acidified prorenin

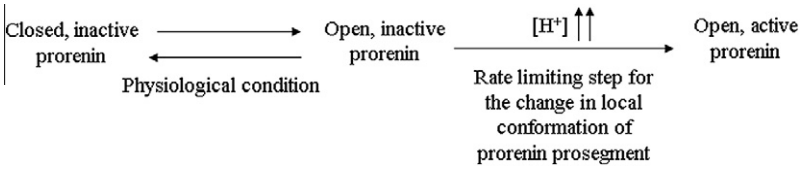
(P)RR-bound form of acidified prorenin at pH 4.5, 5.5, and 6.5 generated the renin activity as shown in Table 1. Under the present assay conditions, we could not observe the renin activity in the immobilized (P)RR incubated with acid-activated prorenin at pH 3.3 because of its large K_D value. The K_m of the receptor-bound native prorenin was 0.50 μM, whereas the apparent K_m values of (P)RR-bound acidified active prorenin at pH 4.5, 5.5, and 6.5 were 0.67, 0.5, and 0.77 μM, respectively (Table 1).

4. Discussion

Prorenin was confirmed to be acid-activated non-proteolytically at pH 3.3, 4.5, 5.5, and 6.5, and its binding properties to the recombinant (P)RR were elucidated in this study. Activation of prorenin under pH 3.3 has been reported to proceed through reversible unfolding of the prorenin prosegment [27]. In this study, reversible non-proteolytic activation of prorenin by acid treatment was verified by Western blot analyses with anti-prosegment antibody as well as anti-renin antibody (Fig. 1A and B). Similar migration distances were observed for the native and acidified prorenin by Western blot analyses and confirmed that prorenin molecules retained their prosegment part even after those acidifications.

Activation of prorenin to a full active renin was reported to have a two-step process [3]. During this process, change in the local conformation of the prorenin prosegment plays the crucial role. Our data, Fig. 1C, suggest that the unfolding of the prosegment occurs partially, and the degree of unfolding depends on the level of pH. We found that prorenin activated at pH 3.3 showed almost 2.5 times higher molecular activity, 10.9 h^{-1} (K_m: 0.5 μM; V_{max}: 14.3 ng Ang-I/ml/h), compared to those of molecules activated at pH 6.5, 4.4 h^{-1} (K_m: 0.33 μM; V_{max}: 5.9 ng Ang-I/ml/h). The possible explanation of this phenomenon of acidified prorenin could be due to the slow release of the product, Ang-I, which might have been hindered by the partially opened prorenin prosegment sequence. Moreover, at physiological condition, a small amount of open, active form of prorenin has been found to be in equilibrium state with that of the predominant inactive, closed form [3] as shown in the equation indicated below:

The equation also indicates that the equilibrium tends to be shifted right side with the increased concentration of hydrogen ions i.e., decreased pH that has ultimately been reflected by the increased activity (Fig. 1C). However, though several studies have



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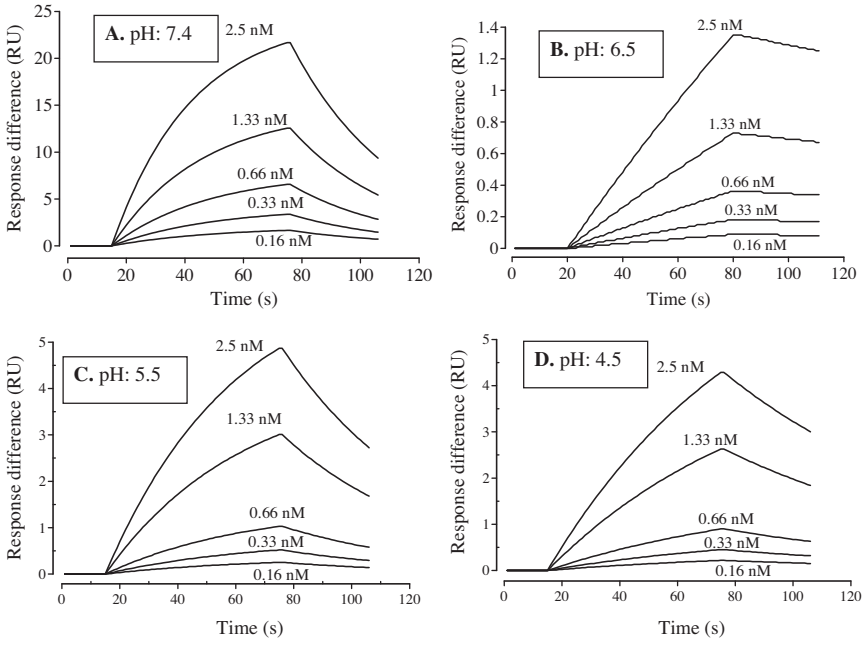

Fig. 2. Real-time binding of acidified prorenin to recombinant human (P)RR by surface plasmon resonance technique in BIAcore assay system. The recombinant (P)RR was immobilized on the surface of CM5 sensor chip through anti-His tag antibody. The apparent dissociation constants (K_D) were determined after evaluating the association and dissociation rate constants simultaneously using Langmuir 1:1 kinetic binding model. Figures A, B, C, and D represent BIAsimulation curve for the binding of prorenin to (P)RR at pH 7.4, 6.5, 5.5, and 4.5, respectively.

Table 1
Binding and activity parameters of the acidified prorenin.

Acid-treated prorenin at	Binding parameters in BIAcore assay system			Binding and activity parameters at equilibrium state		
	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)	K_D (nM)	K_m (μM)	
					Bound state	Free state
pH 7.4	3.6×10^6	0.028	7.8×10^{-9}	1.42	0.50	–
pH 6.5	3.34×10^5	2.67×10^{-3}	8.04×10^{-9}	1.25	0.77	0.33
pH 5.5	9.45×10^5	7.86×10^{-3}	8.29×10^{-9}	2.0	0.50	0.40
pH 4.5	8.45×10^5	0.0119	14.1×10^{-9}	5.0	0.67	0.33
pH 3.3	1.02×10^3	0.0161	1.57×10^{-5}	–	–	0.50

k_a, Association rate constant; k_d, dissociation rate constant; K_D, dissociation constant.

showed that prorenin could be activated non-proteolytically at pH 3.3 [8], this pH does not exist in physiological condition. In the present study, this pH was chosen as the condition of positive control for the acid-activation of prorenin, and its activation level was compared with that observed at pH 4.5 and 6.5. As shown in Fig. 1D, it is suggested that the prosegment part of activated prorenin refolds to its native conformation under the physiological pH. Thereby, such acid-activated and free-form of prorenin possibly participate in Ang-I generation locally in the tissue to modulate the role of RA system.

Prorenin has at least two high affinity binding sites to (P)RR, the decoy (containing the $I^{11P}FLKR^{15P}$, "handle" sequence at the N-terminus of prorenin prosegment) and the "hinge" (the junction between the N- and C-domains of renin/prorenin) [21]. Morales et al. proposed that 11P–26P amino acid containing peptide of prorenin prosegment could achieve stronger binding to (P)RR and also, could be a better inhibitor [28]. Unfolding of the prorenin prosegment sequence due to acidification could ultimately alter the arrangement of these binding sites/regions within the prorenin molecule. However, in this study, partially activated prorenin at pH 4.5–6.5 showed almost similar association rate constant (k_a), dissociation rate constant (k_d), and dissociation constant (K_D) i.e., binding affinity to the (P)RR as it was observed in case of native prorenin at pH 7.4. These findings explain that a regional change possibly occurs around the prosegment in such a manner that the prorenin prosegment still holds flexibility for its binding to (P)RR with similar rate and affinity as the control. On the contrary, at very low pH, for example at pH 3.3, an extremely unexpected behavior of prorenin is observed in terms of its binding to (P)RR having a K_D 10,000 times higher than control (Fig. 2). These data support the possibility that prorenin molecule acidified at such low pH might loss its flexibility. Similar binding pattern of acidified prorenin was observed at equilibrium state as well, although their values of K_D are several folds lower at equilibrium state than those obtained by BIAcore assay system. Those differences can be due to differences of their assay conditions [21]. Therefore, after acid-activated prorenin binds to (P)RR, its additional conformational change can take place to generate 100% of the renin activity.

It is concluded that native conformation and/or spatial local orientation of "handle" region sequence in prorenin prosegment is one of the possibly important factors for the appropriate receptor–ligand interactions. Both prorenin and (P)RR are associated with pathophysiology of many end-stage organ damages related disorders such as diabetic nephropathy, retinopathy and/or hypertension. Some pathological conditions e.g., degranulation of mast cells, diabetic keto-acidosis, and inflammation might lead to decrease local pH that ultimately could increase total levels of activated prorenins. Further, peroxisomes degradation may also decrease local pH. In addition, slightly more acidic pH in tissue interstitial spaces as compared with the circulation has been reported [29]. Thus, taking all these facts under consideration, this research work also carries physiological importance as activated prorenins under acidic condition might modulate local/circulating RA system with/without (P)RR.

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