The Coexistence of Ser84 in Renin and His13 in Angiotensinogen Brings a pH Profile of Two Separate Peaks to the Reaction of Human Renin and Sheep Angiotensinogen

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The pH dependence and kinetics parameters of renin-angiotensinogen reactions were determined using wild-type and S84G mutant human renins and wild-type and H13Y mutant sheep angiotensinogens. It is explained in this report that (i) renin catalyzes acidic and basic reactions of which the optimum pHs are 5.5 and 7.5–8.2 respectively, both of which produce angiotensin I; (ii) Ser84 specific to human renin accelerates the acidic reaction by 75–110% through elevation of $V_{\text{max}}$, and shifts the optimum pH of the basic reaction from 7.5 to 8.0–8.2; and (iii) His13 specific to sheep angiotensinogen accelerates the acidic and basic reactions by 25–42% through reduction of $K_m$. It is concluded from these results that the coexistence of Ser84 in renin and His13 in angiotensinogen brings a pH profile of two separate peaks at pHs 5.5 and 8.2 to the reaction of human renin and sheep angiotensinogen.

Key words: renin; angiotensinogen; pH dependence

Renin (EC3.4.23.15) is the aspartic proteinase that catalyzes the release of angiotensin I (Ang I) from angiotensinogen, playing important roles in the regulation of blood pressure and electrolyte balance.1,2) The catalytic mechanism of renin, however, has not been sufficiently elucidated because of the unique pH profile in the reaction. Renin is generally thought to have neutral optimum pH (pH 6.0–8.0), differently from other aspartic proteinases, which have acidic optimum pH (pH 2.0–4.0) due to two catalytic Asp residues. Strictly speaking, the pH profiles of the renin-angiotensinogen reactions reported to date are various. In this study, we classified them into four types according to their apparent shapes (Fig. 1). The type I pH profile has one major peak at neutral pH with a shoulder at acidic pH, type II one major peak at acidic pH with a shoulder at basic pH, type III two separate peaks at acidic and basic pHs, and type IV a typical bell-shape at acidic pH.

Fig. 1. Classification of pH Profiles of Renin-Angiotensinogen Reactions.

Type I showed one major peak at neutral pH with a shoulder at acidic pH, type II one major peak at acidic pH with a shoulder at basic pH, type III two separate peaks at acidic and basic pHs, and type IV a typical bell-shape at acidic pH.
shoulder at basic pH, e.g., the reaction of human renin with human or rat angiotensigen.\textsuperscript{7,9} The type III profile has two separate peaks at acidic and basic pHs, e.g., the reaction of human renin with sheep angiotensigen.\textsuperscript{5,9} The type IV profile has a typical bell-shape at acidic pH. Very few reports describe this type of pH reaction.\textsuperscript{10,11} The molecular mechanisms involved in the differences in the pH profiles of the renin-angiotensigen reactions have not been identified yet.

In a previous study, we found that only the combination of human renin and sheep angiotensigen gave the type III pH profile for the renin-angiotensigen reaction and that Ser84 specific to human renin contributed to the pH profile, and we speculated that the formation of two hydrogen bonds between Ser84 (the S2 position according to the nomenclature of Schecter and Berger\textsuperscript{12}) and His9 (P2), and between Tyr83 (S3') and His13 (P3'), which is specific to sheep angiotensigen, is important for the unique pH dependence.\textsuperscript{5} However, the possible contribution of His13 of sheep angiotensigen and the role of Ser84 of human renin with respect to enzyme kinetics in pH dependence has not yet been determined.

In this study, we produced H13Y mutant sheep angiotensigen in which His13 was replaced with Tyr, corresponding to the residue of rat and hog angiotensigenes, and determined the pH profiles and kinetics parameters of the reactions of wild-type and S84G mutant renin with wild-type and H13Y mutant angiotensigen in order to determine why only the combination of human renin and sheep angiotensigen gives the type III pH profile for the renin-angiotensigen reaction.

Materials and Methods

Construction of expression plasmid. A plasmid coding for H13Y mutant sheep angiotensigen was prepared as follows: DNA fragments coding for the N-terminal and C-terminal halves of H13Y mutant angiotensigen were amplified by polymerase chain reaction using pcDNA3-sAngn\textsuperscript{13} as a template and oligonucleotide primers CMV-FW with H13Y-RV and H13Y-FW with CMV-RV respectively (Table 1). The N- and C-terminal fragments were digested with SpeI in combination with HindIII and XbaI respectively, and inserted into the HindIII-XbaI cloning site of pcDNA3 (Invitrogen, Carlsbad, CA). The nucleotide sequence of the plasmid was confirmed by DNA sequencing. The resulting plasmid was designated pcDNA3-sAngn-H13Y.

Expression and preparation of recombinant sheep angiotensigenes. A stable cell line producing H13Y mutant sheep angiotensigen was established as described previously.\textsuperscript{14} Briefly, expression plasmid pcDNA3-sAngn-H13Y was cotransfected with a plasmid, pmDHFR, into Chinese hamster ovary (CHO) cells deficient in the dihydrofolate reductase gene. G418-resistant clonal cells were treated with methotrexate (Sigma-Aldrich, St. Louis, MO) to enhance production of the recombinant protein. A stable cell line producing wild-type sheep angiotensigen was described previously.\textsuperscript{15} Culture supernatants of wild-type and H13Y mutant sheep angiotensigen were dialyzed against 20 mM acetate buffer (pH 5.0) and applied to a POROS HS/20 cation exchange column (Applied Biosystems, Framingham, MA) equilibrated with the acetate buffer. Angiotensigen was eluted with a linear gradient of NaCl (160–600 mM) at a flow rate of 8.0 ml/min. Each eluate containing angiotensigen was dialyzed against 10 mM phosphate buffer (pH 7.0). The purified angiotensigenes showed a 56 kDa single band by silver staining after SDS-polyacrylamide gel electrophoresis (data not shown), indicating no contamination by other proteins. The concentrations of wild-type and mutant sheep angiotensigenes were determined by complete digestion with excess renin, as described previously.\textsuperscript{5}

Expression and preparation of recombinant human renins. A stable cell line producing S84G mutant prorenin was established as described above, except that pcDNA3-sAgSP-hPRen-S84G\textsuperscript{5} was used as the expression plasmid. A stable cell line producing wild-type human prorenin was described previously.\textsuperscript{9} Culture supernatants of wild-type and S84G mutant human prorenins were treated with 5 μg/ml of trypsin (Sigma-Aldrich) at 25 °C for 20 min to give active renins. The trypsin reaction was terminated by the addition of a 5-fold concentration of soybean trypsin inhibitor (Type I-S, Sigma-Aldrich). Each preparation was dialyzed against 20 mM acetate buffer (pH 5.0) and applied to a POROS HS/20 column. Renin was eluted with a linear gradient of NaCl (0–600 mM) at a flow rate of 8.0 ml/min. Each eluate containing renin was dialyzed against 10 mM phosphate buffer (pH 7.0). The purified renins showed a 40–43 kDa triplex band by silver staining after SDS-polyacrylamide gel electrophoresis (data not shown), as reported previously,\textsuperscript{14} indicating no contamination by other proteins. The concentrations of wild-type and S84G mutant renins were determined by direct enzyme-linked immunosorbent assay (ELISA) on the assumption that the

<table>
<thead>
<tr>
<th>Designation</th>
<th>Length</th>
<th>Sequence (5'-3')</th>
</tr>
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<tbody>
<tr>
<td>CMV-FW</td>
<td>22 mer</td>
<td>GAGCTCTCGGCTAACTAGAGA</td>
</tr>
<tr>
<td>CMV-RV</td>
<td>22 mer</td>
<td>ACAGATGGCTGGCAACTTAGAAG</td>
</tr>
<tr>
<td>H13Y-FW</td>
<td>24 mer</td>
<td>CACCTACTAGTTAGGTGAAGGACG</td>
</tr>
<tr>
<td>H13Y-RV</td>
<td>24 mer</td>
<td>GTAGACTAGTTAGGTGAAGGAGT</td>
</tr>
</tbody>
</table>

Table 1. Oligonucleotide Primers Used in This Study

Substituted residues are indicated in bold type, and sequences of restriction sites introduced to facilitate efficient construction of the plasmid are underlined.
immunooactivities of the wild-type and mutant human renins were same against the anti-human renin antibody used in this study. Wild-type and S84G mutant human renins were diluted in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6), and coated the wells of a polystyrene microtiter plate (Greiner, Frickenhausen, Germany) for 16 h at 4 °C. The wells were washed with phosphate-buffered saline (pH 7.4) (PBS) containing 1% casein (Sigma-Aldrich), incubated in the same solution for 2 h at 25 °C, and washed with 0.05% Tween 20 in PBS. Rabbit anti-human renin antibody \(^{16}\) (diluted 1:1,000 in PBS) was added to each well. After incubation for 1 h at 25 °C, the wells were washed with 0.05% Tween 20 in PBS. Goat anti-rabbit IgG antibody-peroxidase conjugate (Bio-Rad, Hercules, CA), diluted 1:1,000 in PBS, was added to each well. After additional incubation for 1 h at 25 °C, the wells were washed with 0.05% Tween 20 in PBS. Peroxidase activities in the wells were assayed by the method reported previously.\(^ {17}\)

**Assay of renin activities.** In order to measure pH-dependent activity, wild-type and S84G mutant human renins (17.3 pm) were incubated with wild-type or H13Y sheep angiotensinogen (0.100 μm) in Britton-Robinson buffer at 37 °C for 30 min, as described previously.\(^ {5,9}\) For kinetics analysis, various concentrations of angiotensinogens were used. The Ang I generated was quantified by Ang I-ELISA,\(^ {17}\) and renin activity was expressed as nM of Ang I produced in 1 min. Kinetics parameters were estimated using UltraFit curve-fitting software (Biosoft, Cambridge, UK).

**Identification of the products of the renin-angiotensinogen reactions.** The products of the renin-angiotensinogen reactions at pHs 5.5 and 8.0 were identified by reversed-phase high-performance liquid chromatography. Wild-type human renin (3.2 nm) was incubated with wild-type sheep angiotensinogen (3.8 μm) at 37 °C for 3 h in a reaction mixture (100 μl) containing Britton-Robinson buffer at pH 5.5 or 8.0. The renin reaction was terminated by the addition of 1.9 ml of 0.1% trichloroacetic acid solution. After filtration of the reaction mixture using 30-kDa cutoff membrane device (Amicon Ultra, Millipore, Bedford, MA), the filtrate was applied on a POROS R2/M reversed-phase column (Applied Biosystems), and was eluted with a linear gradient of 0–32% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 6.0 ml/min for 15 min. Synthetic Ang I, angiotensin II, and angiotensin III (Peptide Institute, Osaka, Japan) were used as authentic peptides. Elution of the peptides was monitored by the absorption at 220 nm.

**Statistical analysis.** Kinetics parameters were expressed as mean ± SD, and analyzed statistically by Student’s t test. Differences with probability values less than 5% were considered significant.

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**Results**

**pH dependence of the reactions of wild-type and S84G mutant human renins with wild-type or H13Y mutant sheep angiotensinogen**

As reported previously,\(^ {5,9}\) the reaction of wild-type human renin with wild-type sheep angiotensinogen showed a type III pH profile: two separate peaks, at pHs 5.5 and 8.2 (Fig. 2A), whereas the reactions of S84G mutant human renin with wild-type sheep angiotensinogen (Fig. 2B), wild-type human renin with H13Y mutant sheep angiotensinogen (Fig. 2C), and S84G mutant human renin with H13Y mutant sheep angiotensinogen (Fig. 2D) showed type I pH profiles, each having a major peak at pH 7.5–8.0 with a shoulder at pH 5.5. The activities at individual optimum pHs of these reactions were 0.064, 0.054, 0.047, and 0.040 (nm/ min) respectively.

**Identification of the products of the renin-angiotensinogen reactions at pHs 5.5 and 8.0**

The products at pHs 5.5 and 8.0 were eluted at the same retention time as authentic Ang I from the reversed-phase column, and the amounts of the products were approximately equivalent to the amount of angiotensinogen used as the substrate (380 pmol) (Fig. 3), indicating that both the products were Ang I.

**Kinetics parameters for the reactions of wild-type and S84G mutant renins with wild-type or H13Y mutant angiotensinogen**

The activities of wild-type human renin against wild-type sheep angiotensinogen, S84G mutant human renin against wild-type sheep angiotensinogen, and wild-type human renin against H13Y mutant sheep angiotensinogen were assayed at pHs 5.5 and 8.0 at various concentrations of angiotensinogens. The experimental measurements at each pH agreed well with the Michaelis-Menten’s equation, and kinetics parameters were estimated (Table 2).

At pH 5.5, S84G mutant human renin had a \(V_{\text{max}}\) value 25% lower than wild-type human renin when wild-type sheep angiotensinogen was used as the substrate. H13Y mutant sheep angiotensinogen had a \(K_m\) value 62% higher and a \(V_{\text{max}}/K_m\) value 32% lower than wild-type sheep angiotensinogen when wild-type human renin was used as the enzyme. There were no significant differences among the other kinetics parameters at this pH.

At pH 8.0, S84G mutant human renin had a \(K_m\) value 39% higher and a \(V_{\text{max}}/K_m\) value 28% lower than wild-type human renin when wild-type sheep angiotensinogen was used as the substrate. H13Y mutant sheep angiotensinogen had a \(K_m\) value 30% higher and a \(V_{\text{max}}/K_m\) value 22% lower than wild-type sheep angiotensinogen when wild-type human renin was used as the enzyme. There were no significant differences among the other kinetics parameters at this pH.
Discussion

All the renin-angiotensinogen reactions examined in this study showed biphasic pH dependence (Fig. 2, solid lines). Considering our previous finding that the renin-angiotensinogen reaction occurs at acidic and basic pHs due to two pairs of catalytic residues,9) each pH profile experimentally obtained in this study is thought to be the arithmetic sum of two individual pH profiles, each bell-shaped on the basis of the Michaelis pH function. The

Fig. 2. The pH Dependence of Reactions of Wild-Type and S84G Mutant Human Renins with Wild-Type and H13Y Mutant Sheep Angiotensinogens.

The pH-dependent activities of reactions of wild-type human renin with wild-type sheep angiotensinogen (A), S84G mutant human renin with wild-type sheep angiotensinogen (B), wild-type human renin with H13Y mutant sheep angiotensinogen (C), and S84G mutant human renin with H13Y mutant sheep angiotensinogen (D) were assayed under the condition described in “Materials and Methods.” Solid lines show the experimental measurements, and dotted lines show bell-shape curves conjectured from the solid lines.

Fig. 3. Identification of the Products of the Renin-Angiotensinogen Reactions at pHs 5.5 and 8.0.

The product of the renin-angiotensinogen reaction at pH 5.5 (A), that at pH 8.0 (B), and authentic Ang I (380 pmol) (C) were applied on a reversed-phase column and eluted as described in “Materials and Methods.” Open and solid arrows indicate retention times of authentic angiotensin III (535 s) and angiotensin II (540 s) respectively.
two elementary curves thus conjectured are described with dotted lines in Fig. 2. These curves make it possible to draw the following inferences: The reactions of wild-type human renin with wild-type or H13Y mutant sheep angiotensinogens consist of two reactions, at pHs 5.5 and 8.2 and pHs 5.5 and 8.0 respectively (Fig. 2A and C). Those of S84G mutant human renin with wild-type or H13Y mutant sheep angiotensinogens consist of a major reaction at pH 7.5 and a minor one at pH 5.5 (Fig. 2B and D). Renin catalyzes two reactions, viz., acidic and basic reactions, of which the optimum pHs are 5.5 and 7.5–8.2 and the product is Ang I (Fig. 3).

Replacement of Ser84, which is specific to human renin,5) with Gly, which is common to many renins, decreased the activity of the acidic reaction dramatically (by 52%) and that of the basic reaction moderately (by 15%), and shifted the optimum pH of the basic reaction from 8.2 to 7.5 (Fig. 2A and B) when wild-type sheep angiotensinogen was used as the substrate. Similar phenomena were also observed when H13Y mutant sheep angiotensinogen was used as the substrate (Fig. 2C and D). It is a new finding that Ser84 of human renin can contribute to enhancing the activity of the acidic reaction by 75–110% (Fig. 2B and A; Fig. 2D and C) through elevation of $V_{\text{max}}$ (Table 2). This is probably the main reason only human renin shows the type II pH profile against certain angiotensinogens.

Ser84 (S2) of human renin might interact with His9 (P2) of sheep angiotensinogen through the formation of a hydrogen bond (Fig. 4), as reported previously,5) to give efficient proximity and orientation to the scissile peptide bond of the substrate in the acidic reaction. The hydrogen bond is not likely to contribute substantially to the affinity of human renin for sheep angiotensinogen, because no change was observed in the $K_m$ value of S84G mutant renin at pH 5.5 (Table 2).

![Fig. 4. Model of Interaction between Human Renin and Sheep Angiotensinogen.](image)

The N-flap of human renin (Thr80-Phe91) covers the substrate in the catalytic cleft, in which the catalytic two aspartyl residues (Asp38 and Asp226) face each other. It has not been determined yet which Asp is deprotonated. Two hydrogen bonds, between Ser84 and His9 and between Tyr83 and His13, are formed by the coexistence of Ser84 in renin with His13 in angiotensinogen. At basic pH, Ser84 might directly or indirectly interact with the catalytic residue (Asp or some other) which contributes to the acidic rib of the basic reaction.

### Table 2. Kinetics Parameters for Reactions of Wild-Type and S84G Mutant Human Renins with Wild-Type and H13Y Mutant Sheep Angiotensinogens at pHs 5.5 and 8.0

<table>
<thead>
<tr>
<th>Renin</th>
<th>Angiotensinogen</th>
<th>pH</th>
<th>$V_{\text{max}}$ (nM·min$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}/K_m$ (min$^{-1}$) $\times 10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>5.5</td>
<td>0.139 ± 0.016</td>
<td>0.074 ± 0.014</td>
<td>1.97 ± 0.46</td>
</tr>
<tr>
<td>S84G</td>
<td>WT</td>
<td>5.5</td>
<td>0.104 ± 0.004(*)</td>
<td>0.072 ± 0.016</td>
<td>1.55 ± 0.39</td>
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<tr>
<td>WT</td>
<td>H13Y</td>
<td>5.5</td>
<td>0.160 ± 0.009</td>
<td>0.120 ± 0.007(*)</td>
<td>1.34 ± 0.07(*)</td>
</tr>
<tr>
<td>S84G</td>
<td>WT</td>
<td>5.5</td>
<td>0.147 ± 0.016</td>
<td>0.088 ± 0.008</td>
<td>1.68 ± 0.20</td>
</tr>
<tr>
<td>WT</td>
<td>H13Y</td>
<td>8.0</td>
<td>0.149 ± 0.006</td>
<td>0.114 ± 0.001(*)</td>
<td>1.31 ± 0.04(*)</td>
</tr>
<tr>
<td>S84G</td>
<td>WT</td>
<td>5.5</td>
<td>0.170 ± 0.004</td>
<td>0.120 ± 0.009</td>
<td>1.68 ± 0.20</td>
</tr>
<tr>
<td>WT</td>
<td>H13Y</td>
<td>8.0</td>
<td>0.147 ± 0.008</td>
<td>0.122 ± 0.011(*)</td>
<td>1.21 ± 0.09(*)</td>
</tr>
</tbody>
</table>

The values are expressed as mean ± SD ($n = 4$). Asterisks denote significant difference ($p < 0.05$) against the corresponding parameter for the reaction of wild-type human renin with wild-type sheep angiotensinogen at the same pH.
probably increased the pK_a, which was estimated as the pH giving half of maximum activity, of the catalytic residue involved in the acidic rib of the basic reaction by about 1 pH unit (Fig. 2A and B; Fig. 2C and D). Ser84 might directly or indirectly interact with the catalytic residue, which was not identified in this study (Fig. 4). The distance between the O_y of Ser84 and the Oδ of the catalytic Asp residues is too remote to interact (> 6 Å) in the tertiary structure model of human renin reported by Dhanaraj et al. But renin was crystallized at pH 5.6 for X-ray analysis. Since renin can take different tertiary structures at different pHs, the crystallization of renin at basic pH should provide helpful information to elucidate the reaction mechanism of renin at basic pH.

Replacement of His13, which is specific to sheep angiotensinogen, with Tyr decreased the activities of the acidic and basic reactions, by 30% and 27% respectively, when wild-type human renin was used as the enzyme (Fig. 2A and C). A similar phenomenon was also observed when S84G mutant human renin was used as the enzyme (Fig. 2B and D). The lower K_m values of wild-type sheep angiotensinogen than of H13Y mutant angiotensinogen for human renin (Table 2) suggest that His13 (P^3) of sheep angiotensinogen played a role in elevating renin activity by 25–42% (Fig. 2A and C; Fig. 2B and D) through an increase in affinity for renin. Tyr83 (S^3) of renin is considered to be essential to the catalytic reaction at basic pH. Probably, the Ne of His13 of sheep angiotensinogen makes a hydrogen bond with the O_y of Tyr83 of human renin (Fig. 4), as speculated in a previous study. Nabi et al. reported that substitution of Gln for His13 of sheep angiotensinogen did not change the K_m values of the human renin reaction at pHs 6.5 and 8.5. Probably, their mutant substrate also made a hydrogen bond between the substituted Gln13 of the substrate and Tyr83 of human renin.

The reaction of wild-type human renin with rat angiotensinogen, which has Tyr at position 13, shows a biphasic pH profile, but that with H13Y mutant sheep angiotensinogen did not show the type II pH profile (Fig. 2C). Sheep angiotensinogen must have factor other than His13 to enhance the activity of human renin at basic pH.

It is concluded that the coexistence of Ser84 in renin and His13 in angiotensinogen brings a type III pH profile to the reaction of human renin with sheep angiotensinogen.

References

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