**Note**

**pH-Dependent Inactivation and Reactivation of Recombinant Sheep Angiotensinogen**

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Purified recombinant sheep angiotensinogen (rsAng) lost 74% of the reactivity with human renin during storage at pH 8.0 and 4°C. The inactivated rsAng was reactivated by incubation at acidic pHs. This indicates that pH-dependent inactivation and reactivation occur in rsAng.

Angiotensinogen (Angn) is mainly synthesized in the liver and cleaved by renin (EC 3.4.23.15) to release a decapetide angiotensin I (Ang I). Ang I is processed by angiotensin-converting enzyme (EC 3.4.15.1) to an octapeptide angiotensin II, which causes arteriolar vasoconstriction and stimulates aldosterone release. The renin-Angn reaction is a rate-limiting step in the renin-angiotensin system, and is important in control of blood pressure and electrolyte balance. Biochemical and structural investigations of Angn are essential to gain useful information about this system. However, such investigations have been few because it was very difficult to obtain a large quantity of pure Angn. Sheep Angn is a very useful tool for analysis of human renin reaction because it is a good substrate of human renin. Therefore, we isolated cDNA of sheep Angn and expressed it in COS-7 cells.

We have expressed the gene in Chinese hamster ovary cells and succeeded in a large-scale purification of rsAng. Sixty-one point two mg of pure rsAng were obtained from 6 liters of culture medium. The apparent molecular weight was estimated to be 56,000 on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). The details of expression and purification will be reported elsewhere soon. In this paper, we describe and discuss the pH-dependent inactivation and reactivation of rsAng.

The amount of Angn was calculated from the amount of Ang I liberated after incubation with an excess of recombinant human renin (10 units; 1 unit is defined as the activity that produced 1 μg Ang I/ml/h), 10 mM disopropyl fluorophosphate, 10 mM EDTA, and 100 mM phosphate buffer, pH 6.5, at 37°C for 30 min, on the assumption that 1300 μg of Ang I were liberated from 56,000 μg of rsAng. Ang I was assayed by Ang I ELISA. Recombinant human renin used in this study was prepared as reported previously; the specific activity was 181 μg Ang I/ml/h/mg protein. Specific amounts of rsAng were expressed as mg Angn/mg protein. Protein was measured by the method of Bradford (Bio-Rad Protein Assay kit).

The freshly prepared rsAng had the specific amount of 0.919 mg Angn/mg protein, but this value decreased to 0.236 mg Angn/mg protein after storage of the preparation at 4°C for 8 days in 10 mM Tris-HCl buffer, pH 8.0. It looked as if 74.3% of the rsAng disappeared, but there was no change in total protein concentration and the apparent molecular weight detected on SDS-PAGE (Fig. 1). Furthermore, both the specific amounts of the freshly prepared rsAng and the stored one at pH 8.0 were calculated to be the same value, 0.945 mg Angn/mg protein, when these preparations were incubated with a great excess of renin (448 units) for a longer time (2 h) than the standard assay condition. These results indicated that rsAng did not disappear, but changed from a form of high reactivity to a form of low reactivity during storage at pH 8.0.

To discover whether the rsAng of low reactivity recovered its reactivity after exposure at acidic pHs, the rsAng (114 μg/ml) was mixed with an equal volume of 30 mM citric acid–30 mM potassium phosphate–30 mM boric acid–30 mM barbital–43 mM sulfuric acid buffer adjusted with sodium hydroxide to pH 3.0, 5.0, or 8.0, and

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**Fig. 1.** SDS-PAGE of Purified Recombinant Sheep Angiotensinogen.

The purified rsAng was analyzed by 10% SDS PAGE under reducing conditions. The gels were stained with Coomassie brilliant blue. Lane 1, freshly prepared rsAng (2 μg); lane 2, stored one at pH 8.0 (2 μg).

**Fig. 2.** Reactivation of Sheep Angiotensinogen by Acidification.

Recombinant sheep angiotensinogen stored at pH 8.0 for 8 days was incubated at pH 3.0 (▲) and 8.0 (■) at 4°C.

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*Abbreviations*: Ang I, angiotensin I; Angn, Angiotensinogen; rsAng, recombinant sheep Angiotensinogen; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.
Table Effects of Temperature on Reactivation of Sheep Angiotensinogen

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>pH 3</th>
<th>pH 5</th>
<th>pH 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>277</td>
<td>177</td>
<td>103</td>
</tr>
<tr>
<td>25</td>
<td>680</td>
<td>317</td>
<td>103</td>
</tr>
</tbody>
</table>

* Recombinant sheep angiotensinogen stored at pH 8.0 for 8 days (100%) was incubated at the indicated temperatures and pHs for additional 2 days.

Fig. 3. Reactivation of Reactivated Sheep Angiotensinogen.
Recombinant sheep angiotensinogen was reactivated at pH 5.0 () for 10 days, and then a part of it was incubated at pH 8.0 (○) for additional 3 days.

incubated at 4°C for several days. Reactivity of rsAngn was measured as the amount of Ang I released by renin in this assay: a sample of Angn (100 µl containing 5.7 µg rsAngn) was incubated with 50 µl of recombinant human renin (0.21 units) and 100 µl of 250 mM phosphate buffer, pH 6.5, containing 25 mM disopropyl fluorophosphate and 25 mM EDTA at 37°C for 1 min. As shown in Fig. 2, the reactivity of rsAngn recovered day by day at pH 3.0. The reactivation proceeded faster at pH 3.0 than at pH 5.0, and 25°C than at 4°C (Table). The reactivity of rsAngn that had been restored by the acidification was decreased when the pH was changed from 5.0 to 8.0 in the course of the reactivation process (Fig. 3). These results indicate that rsAngn is inactivated or reactivated depending upon pH.

Printz et al.11 and Hilgenfeldt21 reported the thermal denaturation of Angn; the protein substrate had a marked loss in reactivity with renin over a very narrow temperature range. The thermally induced loss of activity was irreversible, but the pH-dependent inactivation described here was reversible. Therefore these inactivations must be caused by different changes in the three-dimensional structure of Angn.

Benkoulouche et al.27 reported the crystal structure and conformational analysis of angiotensinogen fragments, and suggested that acetyl-histidyl-prolyl-phenylalanyl-histidyl-methyl amide, which is the 6–9 fragments of Ang I, preferred to take a β-turn structure dominated by intramolecular hydrogen bonding. On the other hand, Dealwis et al.8 reported X-ray analysis of renin complexed with a decapptide inhibitor that is based on the 4–16 fragments of rat angiotensinogen; the inhibitor was attached in extended form to the enzyme. These two reports indicate a possibility that the extended form of the Ang I fragment is preferable for renin, but the folded form (β-turn structure) is not.

The three-dimensional structure of ovalbumin,91 of which the amino acid sequence was similar about 50% to that of sheep Angn, indicated that β-structure motifs are found across the molecule. Thus Angn is also expected to have the β-structure supporting the three-dimensional structure. Circular dichroism analysis of rat Angn showed that rat Angn contained 37% β-structure at neutral pH, and the content decreased at a basic pH. The molecule shifted into a more ordered conformation at an acidic pH and the amount of irregular structure increased at pH 9.5.21 A pH-dependent change in the β-structure may affect the reactivity of Angn.

Our findings described here are of great importance for measurement of renin activity. It is necessary to pay attention to pH-dependent changes in the conformation or reactivity of Angn.

References