Structure of the Mouse Submaxillary Gland Renin Precursor and a Model for Renin Processing
Arthur C. Corcoran Memorial Lecture

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SUMMARY The complete amino-acid sequence of mouse submaxillary gland preprorenin is reported, and a model presented for the maturation of preprorenin into prorenin and the mature active form of renin. Properties of prorenin, renin, and other aspartyl proteases are compared. The biological significance of prorenin and its relationship to inactive renin are explored.

KEY WORDS • amino acid • preprorenin • prorenin • renin • submaxillary gland • molecular cloning • nucleotide sequence • ribonucleic acid

THE structure of renin has been the subject of intense interest for many years. However, the extremely low concentration of renin in animal and human kidney has prevented its purification in amounts sufficient to determine its entire amino-acid sequence. Renin from the mouse submaxillary gland has physicochemical, enzymatic, and immunological properties similar to renal renin1,2 and therefore constitutes an excellent alternative for the study of renin structure since it represents as much as 5% of the total protein content of this gland. Whereas conventional amino acid sequencing permits the determination of the primary structure of a protein, the techniques of molecular biology enable the determination of the nucleotide sequence of the mRNA coding for a protein.3 From the nucleotide sequence, the amino-acid sequence of biosynthetic precursor forms of the protein can be deduced. The molecular cloning of mouse submaxillary renin cDNA4 has allowed the determination of the aminoacid sequence of the renin precursor.

The present study is the result of a collaboration between the Unite de Genetique et Biochimie du Developpement (Institut Pasteur) and the Unite de Pathologie Vasculaire et d'Endocrinologie Renale (Institut National de la Sante et de la Recherche Medicale). The complete amino-acid sequence of mouse submaxillary gland preprorenin is reported. A model for the maturation of preprorenin into prorenin and the mature active form of renin, and a comparison among prorenin, renin, and the other aspartyl proteases are presented. Finally, the question of the biological significance of prorenin and its relationship to "inactive" renin is discussed.

Molecular Cloning and Nucleotide Sequence of Mouse Submaxillary Gland Renin cDNA

The amount of mouse submaxillary gland renin is under both genetic and hormonal control. Mice from some inbred strains (Swiss, AKR, ...) have high renin activity and other strains (BALB/C) have low activity with intermediate levels in the F1 hybrids.5,6 In both high and low renin strains, submaxillary gland renin is much higher in males than females due to the effects of androgens.7 These differences in the expression of the renin gene(s) in the mouse submaxillary gland were exploited in the isolation of renin mRNA and the identification of recombinant clones containing renin cDNA.4 RNA was extracted from the submaxillary gland of male Swiss mice, and poly (A)-containing mRNA was purified by oligo (dT)-cellulose affinity chromatography. In vitro translation of mRNA was performed using the mRNA-dependent rabbit reticulocyte lysate translation system; renin precursor was immunoprecipitated and characterized as a 45,000 dalton protein on SDS-polyacrylamide gel electrophoresis. The mRNA coding for renin was then purified by sucrose density gradient ultracentrifugation. The renin precursor appeared to be the main polypeptide encoded by the 18 S mRNA species. Purified mRNA was transcribed into cDNA, converted into double-stranded cDNA, and inserted into the Pst I site of pBR 322 by the dC/dG tailing procedure.
The recombinant clones were selected by differential genetic screening: the recombinants containing the renin copy were able to hybridize with renin cDNA transcribed from the male Swiss mRNA but not with cDNA transcribed from the low renin-producing mice (female Swiss and male BALB/c). The recombinant clones were further selected by the technique of hybrid-arrested translation. A recombinant clone pRn 5-3 containing a 1100-base pair insert was identified and was used to study the expression of renin mRNA in the submaxillary gland and in the kidney of different mouse strains. RNA prepared from kidney and submaxillary glands of both high renin and low-renin producing strains was denatured by glyoxal dimethyl-sulfoxide, subjected to agarose gel electrophoresis and then blotted on nitrocellulose paper. Hybridization was performed with 32P-labelled pRn 5-3. Renin mRNA from both submaxillary gland and kidney had a similar length of 1600 nucleotides.

By nucleotide sequence, it was found that clone pRn 5-3 contained a poly(A) extension, corresponding to the 3' terminal portion of renin mRNA. Later, a clone designed pRn 4-7 (1250 nucleotides in length) was isolated, containing a sequence 5' upstream of pRn 5-3 cDNA. As clones pRn 5-3 and pRn 4-7 shared an identical segment of about 900 nucleotides, together they represented an essentially complete transcript of renin mRNA. The nucleotide sequence of these cDNAs was determined by the method of Maxam and Gilbert. The sequence strategy and the nucleotide sequence of mouse submaxillary renin mRNA, as deduced from the sequence of these two plasmids, has been reported by Panthier et al. The amino-acid sequence is that deduced from the nucleotide sequence (fig. 1).

N-Terminal Sequence of Pure Active Renin Extracted from Mouse Submaxillary Gland

To establish the relationship between the renin precursor and the mature active enzyme, the N-terminal sequence of pure active renin was determined. Renin was purified from the submaxillary glands of male AKR mice. Special care was taken to avoid proteolytic degradation. The submaxillary glands were immediately placed in liquid nitrogen after excision and then homogenized at 4°C in a citrate-phosphate buffer, 0.1 M, pH 4.5, containing the following protease inhibitors: EDTA (5 mM), N-ethylmaleimide (5 mM), diisopropylfluorophosphate (0.1 mM), benzamidine (5 mM), and aprotinine (4 x 10^-6 UIP/1) (Buffer A). These inhibitors were added to all buffers throughout the purification. The homogenate was centrifuged at 15,000 g for 30 minutes, and renin was precipitated from the supernatant with 4.2 M ammonium sulfate.

![Sequence Diagram](http://hyper.ahajournals.org/)

**Figure 1.** Entire amino-acid sequence of mouse submaxillary renin, as deduced from the nucleotide sequence of submaxillary renin cDNA. Indicated in squares are the signal peptide and the profragment sequences (see model for renin processing, figure 3).
The precipitate was dialyzed against Buffer A and submitted to affinity chromatography on a column of CBL-pepsstatin-aminohexyl-agarose. Renin was eluted with a phosphate buffer, 0.1 M, pH 7.5, containing 2 M urea. The renin peak was dialyzed against phosphate buffer 0.1 M, pH 7.0, containing 0.3 M NaCl (Buffer B). The dialysate was submitted to a final purification by high performance liquid chromatography (HPLC). HPLC was performed on a Toyoda Soda TSK SW 300 column at a flow rate of 1 ml/min with Buffer B. Renin was monitored by its absorbance at 280 nm and by its enzymatic activity, using synthetic N-acetyl-tetradecapeptide as a substrate.

Renin appeared homogeneous on analytical HPLC and SDS-gel electrophoresis (fig. 2) with a MW of 38,000, which agreed well with that of 36-37,000 found by Cohen et al. However, after reduction by mercaptoethanol, the molecular weight shifted to 35,000, and a faint band appeared at the front of the gel. This result suggested that renin consisted of two chains linked by a disulfide bridge. After reduction, the two chains were 14C-carboxymethylated and separated by Sephadex G100 gel filtration in the presence of 5 M guanidine hydrochloride (fig. 2). Peak A corresponded to the main protein band seen on SDS-gel electrophoresis (MW 35,000) and Peak B to the minor band. The presence of cysteine residues in the two chains was shown by the coincidence between the optical density and the radioactivity of the 14C-carboxymethylcysteines. That renin had two polypeptide chains linked by a disulfide bridge was independently found by Misono and Inagami. The two polypeptides were submitted to automatic Edman's degradation; their N-terminal sequences are shown in table 1.

### Table 1. Amino-Terminal Sequence of the Renin Chains A and B Determined by Automated Edman Degradation

<table>
<thead>
<tr>
<th>Chain</th>
<th>Amino-acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chain A</strong></td>
<td>Ser - Ser - Leu - Thr - Asp - Leu - Ile - Ser - Pro - Val - Leu - Thr - Asn - Tyr - Leu - Asn - Ser - Glu - Tyr - Val</td>
</tr>
<tr>
<td><strong>Chain B</strong></td>
<td>Asp - Lys - Leu - Cys - Thr - Val - Ala - Leu - His - Ala - Met - Asp - Ile - Pro - Pro - Pro - Thr - Gly - Pro - Val - Trp</td>
</tr>
</tbody>
</table>

FIGURE 2. Purification of renin and separation of the two chains. SDS-gel electrophoresis is shown in the inset, where lane 1 = 15 pg of renin reduced by incubation with 1% SDS and 5% mercaptoethanol for 10 minutes at 100°C; lane 2 = 15 pg of nonreduced renin; and lane 3 = protein markers. At the lower part of the figure, the separation of the two renin chains after reduction and carboxymethylation with 14C-labelled iodoacetamide was performed on Sephadex G100 and the peaks were monitored by their absorbance at 280 nm (open circles) and by counting each fraction in a scintillation counter (closed circles). Peaks A and B were clearly separated and correspond to chains A and B of renin.
Model for Renin Processing

Comparison of the amino-acid sequences obtained from the pure protein and that deduced from the coding region of renin mRNA suggests a model for the maturation of the renin precursor.

The N-terminal sequence of peptide A coincides with the portion of preprorenin starting at position 64 and the sequence of peptide B coincides with the portion of the enzyme starting at position 354. The proposed model for renin processing (fig. 3) involves at least two separate processing events.

1. A signal peptide (presequence) is first cleaved off by membrane processing enzymes to produce prorenin. The exact length of the signal peptide is not known since the N-terminus of the prorenin (putative inactive renin, see below) was not determined. Examination of the structure of the renin signal peptide revealed characteristics common to other described signal peptides: a region of charged amino acids, followed by a region rich in hydrophobic amino acids. The endopeptidase called signal peptidase splits the peptide bond on the carboxy-terminal side of uncharged amino acids with small side chains. In addition, the residues located in the neighbourhood of the cleavage site exhibit a high potential for making $\beta$-turns or aperiodic conformations. Therefore, the cleavage site for preprorenin might occur after cysteine 18 but also might occur after serine 21 or glycine 25.

2. The prorenin is then subject to two other cleavages leading to active mature renin, containing two chains linked by a disulfide bond. Each of the proteolytic cleavages occurs after a dibasic peptide: Lys$^{352}$-Arg$^{353}$ and Arg$^{352}$-Arg$^{353}$. This mechanism of peptide maturation is similar to that observed in cases of prohormone processing where the active hormone is released from an inactive precursor by cleavage after a pair of basic amino-acid residues. The exact length of the A and B chains of renin could not be determined in our study since the carboxyl terminus of the two chains was not determined. However, we speculated that an extensive processing at the carboxyl terminus was unlikely because of the similarity between the molecular size found for chains A and B, and that calculated from the deduced amino-acid sequence. Since this study was published, Misono et al. have reported the complete amino-acid sequence of active mouse submaxillary gland renin. According to our numeration, they found Asn$^{351}$ and Arg$^{350}$ at the carboxyl terminus of chains A and B respectively. Their study indicates that there is no processing at the C-terminus of chain B, and that a further proteolytic cleavage occurs in chain A after Asn$^{351}$, leading to the removal of the Arg$^{352}$-Arg$^{353}$ pair. A similar maturation event has also been reported for the vasopressin precursor where the dibasic peptide Lys-Arg is cleaved from the C-terminus of vasopressin.

In the model proposed, chains A and B are linked by a disulfide bridge occurring between Cys$^{357}$ and Cys$^{320}$. This model is based on the conservation of cysteine residues within the aspartyl-protease family. Similarly, it is possible that there are two intrachains disulfide bridges between Cys$^{314}$ and Cys$^{321}$, and between Cys$^{227}$ and Cys$^{231}$.

Comparison with Aspartyl-Proteases

Similarities

Studies performed on the inactivation of renin by diazo-compounds and 1,2-epoxy-3-(p-nitrophenoxy)
propane led to the suggestion that renin belonged to the aspartyl-protease family, like pepsin, chymosin, penicillopepsin, and cathepsin D. Indeed, when the amino-acid sequences of these proteins are aligned with the renin sequence so as to maximize the homology between the proteins, a strong homology is found. The sequence identity between active renin and prointrinsic pepsin was 43% and 37% when the entire molecules of prorenin and porcine pepsinogen were compared. For all members of the aspartyl protease family, the highest homology is found in the region of the two aspartyl residues involved in the catalytic site: Thr-Gly-Ser and Asp-Thr-Gly is found in all the aspartyl proteases sequenced so far. Furthermore, the two residues Tyr and Trp of penicillopepsin, which are thought to participate in the catalytic mechanism, are conserved, occurring respectively at positions 146 and 108 of the prorenin chain. Finally, the conservation of the position of the cysteine residues and the high structural homology with the other aspartyl proteases both suggest that the tertiary structure of renin might be similar to that of pepsin and penicillopepsin.

X-ray studies of aspartyl proteases have shown that these proteins possess a bilobal structure with a well-defined cleft. The two aspartyl residues of the catalytic site lie in close proximity on either side of the cleft. Tang et al. have proposed that these enzymes evolved by gene duplication of an ancestral gene coding for a protein of about 150 residues with a tertiary structure similar to one lobe of pepsin. According to their hypothesis, after gene duplication, the two subunits evolved divergently with a subsequent gene fusion which was presumably advantageous in assuring the correct association of the dissimilar units. In the case of mouse submaxillary renin, no three dimensional structure is yet available although renin crystals have been obtained. However, the availability of a nucleotide sequence encoding an acid protease allows further comment on this hypothesis. As shown in figure 4, nucleotide and amino-acid sequences between positions 331 to 376 (corresponding to amino acids 98 to 112) and positions 886 to 931 (corresponding to amino acids 283 to 296) possess a 56% homology in their nucleotide sequence and a 40% homology in their amino-acid sequence. The presence of such an homology supports the theory that the two lobes of acid proteases have evolved by duplication of a primordial gene encoding a polypeptide chain corresponding to a single lobe unit. If the gene encoding renin contains an intron, one would expect this to occur between the segments of the gene which encode the two domains of the enzyme.

### Differences

There are interesting differences between renin and other aspartyl proteases, which are summarized in table 2. Bovine and porcine pepsinogens and bovine prochymosin are converted into active enzymes by cleavage between two uncharged amino acids to release the amino-terminal "activation peptide" (fig. 5). For porcine pepsinogen, 44 amino acids are removed, and Ile is generated as the amino terminus of active pepsin. In contrast, the processing of submaxillary prorenin into active renin involves a serine esterase-like cleavage at the carboxyl side of pairs of basic amino acids, leading to the formation of the A and B chains. This mechanism of processing may be peculiar to the submaxillary gland where serine esterases are abundant, especially since kallikrein, a serine esterase, has been found in the same granules as renin. Another theoretical site of cleavage of prorenin could be located between Leu and Ile. Whether kidney renin is processed in the same manner as that postulated for submaxillary renin has not yet been determined. The mechanism of activation of porcine pepsinogen into pepsin is pH-dependent. At pH 3 and below, intramolecular activation occurs, whereas above this pH value the activation involves the intermolecular action of newly formed pepsin. That similar intra- and intermolecular activation mechanisms may occur for prorenin is suggested by the recent preliminary report of Kokubu and Hiwada. They proposed that inactive human renin can be activated by the addition of active...
TABLE 2. Comparison among Submaxillary Renin and Other Aspartyl Proteases (Hog and Bovine Pepsins; Prochymosin)

<table>
<thead>
<tr>
<th></th>
<th>N-Terminal pro fragment (amino acids cont.)</th>
<th>Peptide bond cleaved</th>
<th>Activation</th>
<th>Inhibition of enz. act. by activation peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hog pepsin</td>
<td>44 Leu-Ile</td>
<td></td>
<td>Intra molecular activation at acidic pH</td>
<td>peptides 1-16</td>
</tr>
<tr>
<td>Bovine pepsin</td>
<td>45 Leu-Val</td>
<td></td>
<td></td>
<td>peptide 1-17</td>
</tr>
<tr>
<td>Prochymosin</td>
<td>44 Phe-Gly</td>
<td></td>
<td></td>
<td>?</td>
</tr>
<tr>
<td>Submaxillary renin</td>
<td>40 Arg-Ser</td>
<td>Trypsic-like cleavage</td>
<td></td>
<td>peptide 5-12</td>
</tr>
</tbody>
</table>

PRORENIN
(RENINOGEN)  
Phe-Thr-Lys-Arg-Ser-Ser-Leu-Thr-Asp-Leu-Ile-Ser-Pro

BOVINE
PEPSINOGEN  
Lys-Tyr-Ile-Arg-Glu-Ala-Ala-Thr-( )-Leu-Val-Ser-Glu

HOG
PEPSINOGEN  
Lys-Tyr-Phe-Pro-Glu-Ala-Ala-Ala-( )-Leu-Gly-Gly

BOVINE
PROCHYMOSIN  
Lys-Tyr-( )-Arg-( )-Gly-Phe-Gly-Glu-Ala-Ser

Figure 5. Sequence of the cleavage site for activation of prorenin, bovine and hog pepsinogens, prochymosin. The arrow indicates that the cleavage site in the prorenin is located after Lys-Arg. In the case of other aspartyl proteases, the cleavage site is located after an uncharged amino acid. Homologous amino acids with renin are underlined.

human renin. Clearly, more data must be obtained using highly purified prorenin before one can be certain that this mechanism of intermolecular activation applies to prorenin.

Another major difference between renin and other aspartyl proteases is its higher catalytic optimum pH and its extreme substrate specificity. Comparison of the three-dimensional structures of these enzymes will allow definition of the amino-acid residues involved in substrate binding and in the catalytic center. This information will also be extremely helpful for the design of specific and potent renin inhibitors.

Is Prorenin Inactive Renin?

Several investigators have reported the presence of inactive renin in both human plasma and in human kidney. Inactive renin can be activated by exposure to cold, by acid treatment, and by partial proteolysis. The serine esterases (trypsin, kallikrein, plasmin, tonin, ...) are particularly effective activators of inactive renin. Since inactive human renin has not yet been sequenced, it is still speculative to name it "pro-renin," and its relationship to the prorenin described in the present report remains to be determined.

During the course of purification of submaxillary renin in the present studies, no inactive form of renin was found, although great care was taken to inhibit protease activity. Similarly, Nielsen et al. were unable to detect an inactive form of renin in submaxillary gland extracts. These authors homogenized the submaxillary glands in the presence of renin antibodies so as to protect the renin from proteolytic enzymes and measured renin by both direct radioimmunoassay and enzymatic activity. Using the same methodology, this group was similarly unable to detect the presence of inactive renin in mouse kidney. It is therefore likely that mouse prorenin is immediately processed into active renin by intracellular enzymes. Consequently, the renin granules observed by electron microscopy of the submaxillary gland must contain mature active renin. Similar processing events have been described in other secretory cells where the packaging of the peptide hormones is usually associated with their processing.

We have attempted to determine the relationship between prorenin and inactive renin by a different
experimental approach. It has been shown that both porcine\(^2\) and bovine\(^3\) pepsins are inhibited by the N-terminal fragments of their corresponding pepsinogens. Such fragments exhibit a high affinity for pepsin with IC 50% around 10\(^{-4}\)M. By structural analogy, a peptide derived from the renin profragment (amino acid residues 20 to 37) was synthesized and shown to inhibit submaxillary renin with an IC 50% of 2 \times 10\(^{-4}\)M (G. Evin et al., unpublished data). This experiment, taken together with the other similarities between the pepsinogen and prorenin structures discussed, strongly suggests that prorenin is enzymatically inactive. However, given the failure to detect inactive renin in the mouse, any speculation that prorenin and inactive renin represent the same molecule must be very tentative.

The model for renin processing presented in this paper is based on the amino-acid sequence deduced from the nucleotide analysis of the coding region of mouse submaxillary renin mRNA and its comparison with the structure of the mature active form of renin. This model is supported by the analogy that exists between prorenin and the other aspartyl proteases, whether this model is true. The model for renin processing presented in this paper is based on the amino-acid sequence deduced from the nucleotide analysis of the coding region of mouse submaxillary renin mRNA and its comparison with the structure of the mature active form of renin. This model is supported by the analogy that exists between prorenin and the other aspartyl proteases, whether this model is true.

### References

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