Production and Characterization of Human Renin Antibodies with Region-oriented Synthetic Peptides*

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Polyclonal and monoclonal antibodies were raised against pure human renin, but nothing was known about the regions against which they were directed. Using a three-dimensional model of mouse submandibular renin, we selected seven peptide sequences as belonging to potential epitopes. The main criteria for their choice were the location of the peptide sequences near the catalytic region and on the surface of the renin molecule and their hydrophilicity. After transposition of the regions to the 340-amino acid sequence of human renin, the seven peptides (corresponding to amino acids 50-60, 63-71, 81-90, 118-126, 162-169, 247-255, and 287-295) were synthesized, coupled to bovine serum albumin, and injected into rabbits.

Five of these peptides elicited antibodies, and 50-68% binding of the corresponding iodinated peptide was obtained with a 1:25 dilution of antiserum. The antisera titers ranged from 1:5,000 to 1:100,000 when tested by enzyme-linked immunosorbent assay. The same antisera bound 15-65% of labeled pure human renin at a final dilution of 1:2.5, the highest percentage being obtained with peptide 81-90 antiserum. At a 1:5 dilution, the five antisera inhibited renin activity by 23-68% in human plasma with a high renin activity (40 ng of angiotensin I/h/ml). At a final dilution of 1:50, peptide 81-90 antiserum was still capable of producing 25% inhibition. Purified IgG (0.6 mg) from this antiserum inhibited pure human renin activity by up to about 40%, as measured by its reaction with pure synthetic human tetradecapeptide substrate. Antigenic peptides that mimic a part of the human renin sequence, especially peptide 81-90 representing the "flap" covering the cleft between the two renin lobes, constitute promising tools for the development of a synthetic antirenin vaccine.

Renin (EC 3.4.23.15), an aspartyl protease, plays a key role in regulating blood pressure homeostasis. It cleaves its substrate, angiotensinogen, to produce angiotensin I which is then converted by the action of a dipeptidyl carboxypeptidase into angiotensin II, a potent vasoconstrictor.

Polyclonal and monoclonal antibodies have been raised against the entire molecule of renin and found to be useful tools for biochemical and physiological studies of this enzyme (1-5). However, there are no data on the epitopes toward which these antibodies are directed (2). Recent studies showed that synthetic peptides mimicking a protein segment can be used as immunogens to elicit antibodies which react with the parent molecule (6). For example, antibodies raised against synthetic peptides of the VP1 polypeptide of the foot-and-mouth-disease virus are able to produce antibodies which protect animals against subsequent challenge with the virus (7).

The primary structure of human renin was recently elucidated from the structure of its cDNA (8), but renin has not yet been crystallized, and its tertiary structure has not been determined directly. A three-dimensional model of mouse submandibular renin has, however, been derived (9); and since human renin has a 68% homology with mouse renin (8), we used this model to select peptides which could be potential epitopes of human renin. The final goal was to produce, against synthesized peptides, antibodies capable of recognizing the entire renin molecule and of inhibiting its enzymatic activity.

MATERIALS AND METHODS

Selection of Peptides

The choice of peptides was based on coordinates in a model of mouse submandibular renin generously supplied by Dr. T. Blundell. The model was displayed on a three-dimensional graphic console PS 300 (Evans and Sutherland) using the Manosk Program (10). The regions were transposed to human renin by aligning the sequences of mouse and human renin (8). The peptides were selected after taking into consideration regions near the active site which were located on the surface of the renin molecule and were peculiar to human renin. Seven peptides predicted to belong to such epitopes are shown in Fig. 1. They were named according to the numbering of mature human renin (8).

Of these peptides, sequence 81-90 corresponds to the "flap" segment which holds the substrate in the catalytic site (9). Sequences 118-126 and 247-255 are situated on the edges of the cleft which is located between the two lobes of renin and leads to the catalytic site. The three segments 50-60, 63-71, and 162-169, which protrude from the molecule, were chosen for their particular structures. Thus, peptide 50-60 possesses a natural disulfide bridge loop which stabilizes its conformation, peptide 63-71 contains hydrophilic seryl residues, and peptide 162-169 contains the Asp-Ser-Glu segment which is peculiar to human renin and contributes to the specific requirement of human angiotensinogen for primate renins (11). The last peptide, 287-295, corresponds to the region where the A and B chains of mouse submandibular renin are cleaved (12).

As peptide sequences 63-71 and 118-126 do not include a tyrosyl residue, an extra tyrosyl was added to the NH₂ terminus to allow the peptide to be iodinated. This is why they are named Y-63-71 and Y-118-126, respectively. The sequences of the seven peptides synthesized are shown in Fig. 1.
Ac-Lys-Cys-Ser-Arg-Leu-Tyr-Thr-Ala-Cys-Val-Tyr-Oh 50-60
Tyr-Leu-Phe-Asp-Ala-Ser-Asp-Ser-Ser-OMe  Y-63-71
Leu-Arg-Tyr-Ser-Thr-Gly-Thr-Val-Ala-Gly-Oh 81-90
Tyr-Pr-Pro-Met-Leu-Ala-Glu-Asp-Gly-NH2  Y-118-126
Tyr-Aas-Arg-Asp-Glu-Ala-Ser-NH2 162-169
Gly-Ala-Lys-Cys-Arg-Leu-Phe-Ser-Tyr-NH2 247 255
Gln-Glu-Ser-Tyr-Ser-Ser-Lys-Leu-NH2 287-295

FIG. 1. Sequences of the seven synthetic peptides chosen as potential renin epitopes. Numbering refers to the primary structure of human renin (640 amino acids). Y indicates a tyrosyl residue added to the native structure.

Peptide Synthesis

Peptide Y-63-71 was synthesized in the liquid phase by a stepwise strategy using t-butyloxy carbonyl for temporary N-protection, the benzyl group for side chain protection, and the benzotriazoly-N-oxytrisdimethylammonophosphonium coupling reagent (13). The COOH terminus was esterified with methanol to mask the ionizable carboxyl. The final deprotection was performed by hydrolysis, and purification was by reverse-phase HPLC.3

All peptides except sequence 63-71 were synthesized by conventional solid-phase peptide synthesis on different functionalized polystyrene beads; substitution on peptide-resins was determined with a picric acid test (14). The coupling steps were performed with benzotriazoly-N-oxytrisdimethylammonophosphonium reagent diisopropylphosphamide to bring the pH into range 7-8 and 2 eq of t-butyloxycarbonyl-amino acid (13). HF cleavage was used for final deprotection.

In peptide 50-60, the disulfide bond was obtained in a solution containing 400 mg of the crude free peptide in 500 ml of water using carboethoxysulfenyl chloride (15) as described previously (16). Carbontriazoly-N-oxytrisdimethylammonophosphonium reagent diisopropylphosphamide to bring the pH into range 7-8 and 2 eq of t-butyloxycarbonyl-amino acid (15). HF cleavage was used for final deprotection.

The peptides were purified in one step by preparative reverse-phase HPLC (C18 column, Whatman ODS3, 25 × 500 mm, 10 μm). Isocratic elution was performed in 0.1% trifluoroacetic acid/methanol with the percentage of methanol adjusted to the peptide. The peptides were characterized by analytical HPLC, 390-MHz two-dimensional 1H nuclear magnetic resonance (Bruker), and amino acid analysis.

Coupling of Peptides to Carrier Protein and Antibody Production

Synthetic peptides were coupled to bovine serum albumin (Sigma) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide for Y-63-71 and glutaraldehyde for the other peptides (18, 19). Coupling efficiency was 25% with the former agent and 50-80% with glutaraldehyde, as estimated by binding of a tracer amount of the 125I-labeled peptide added to the reaction mixture.

Albumin-conjugated peptides (50 μg) in complete Freund's adjuvant were injected intradermally into three to six male Fauve de Bourgogne rabbits (20). Injections were repeated every 3 weeks with incomplete Freund's adjuvant. Blood samples were collected 10 days after the third and fourth injections.

Results of Peptide to Renin Antibodies

The binding of synthetic peptides to renin antibodies was tested byRIA and ELISA using eleven polyclonal antibodies (R11, R12, R14, R16, R21, R22, R24, R25, R26, Donc, and Bof) (21) and eight monoclonal antibodies (F15, 3A12, 2D12, 4E1, 3E5, 4B11, 4G1, and R3-36-16) (2) raised against pure human renin, as previously reported (1, 5). Peptides were radiolabeled by the chloramine-T method (23). In the case of peptide 50-60, IOD0-GEN was used according to Fraker and Speck (24). Free 125I was separated from the peptide using a Sep-Pak C18 cartridge (Water Associates) (25). The retained peptide was eluted with 40% acetonitrile in 0.1% trifluoroacetic acid/triethylamine buffer, pH 2.5.

For RIA, 5000 cpm of iodinated peptide was incubated at 4°C for 24 h with 200 μl of pure or 1:10 diluted human renin polyclonal or monoclonal antibody in a final volume of 500 μl with 0.1 M phosphate-buffered saline (PBS), pH 7.5. The bound peptide was precipitated with 1 ml of 20% polyethylene glycol (M, 8000) in the same buffer, which also contained 1 mg of bovine γ-globulin (Sigma).

For ELISA, plates were coated with 5 μg of the peptide in 0.1 M Tris-HCl, pH 8.6. After extensive washing, 1% bovine serum albumin in PBS at pH 7.4 was added to saturate the nonspecific binding sites. After washing, either human renin antiserum at a dilution of 1:500 or 1:100 μg of monoclonal antibody was added, and incubation was allowed to proceed for 30 min at 37°C. Plates were washed, and the bound antibody was detected and quantified with alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse γ-globulin using p-nitrophenyl phosphate as a chromophore. The colorimetric reaction was read at 405 nm on a TiterTek Multiskan Photometer (Flow Laboratories). A background value (A0) was obtained from wells not coated with the peptide. Negative controls involving peptides and preimmune rabbit serum were incorporated. An A/A0 ratio of 2.0 or more was interpreted as significant binding of antibody to peptide.

Titration of Peptide Antibodies

The antibodies directed against the different peptides were titrated by both RIA and ELISA.

In RIA, the titer of the peptide antiserum was defined as the dilution of serum that gave 50% binding of tracer. A tracer amount of peptide (5000 cpm) was incubated at 4°C for 24 h with 200 μl of peptide antibody, diluted and coated PBS containing 1:10 and 1:100 in 500 μl final volume of 0.1 M PBS, pH 7.5. The bound fraction was precipitated as above with 20% polyethylene glycol.

In ELISA, the titer represented the maximal dilution of antiserum which allowed a positive signal versus the control with peptide antiserum at dilutions of 1:10 to 1:100,000, as described above.

Binding of Peptide Antibodies to Human Renin and Inhibition of Its Enzymatic Activity

Three methods were used to test the ability of the peptide antibodies to recognize renin.

Binding to 125I-Renin—A tracer amount of human renin (5000 cpm) was incubated at pH 7.5 for 24 h at 4°C either with peptide antiserum diluted 1:2.5 to 1:100 or with the corresponding IgG purified on protein A-Sepharose (26). The bound fraction was precipitated with polyethylene glycol as described above.

Inhibition of Plasma Renin Activity—The ability of the peptide antibodies to inhibit renin enzymatic activity was tested on a pool of human plasma having a high renin activity (40 ng of angiotensin I/ml). Plasma (25 μl) was preincubated at 4°C overnight with 100 μl of antiserum in PBS, pH 7.5, containing 1% EDTA (final volume 0.2 ml). Next, an excess of plasma renin substrate (200 pmol) was added in order to ensure zero-order behavior, and the mixture was incubated for 30 min at 37°C in PBS, pH 5.7. The final dilution of the antiserum was 1:5 to 1:50. The angiotensin I generated was measured by RIA (27). A blank was included using the same dilutions of the corresponding antiserum.

Inhibition of Hydrolysis of Human Tetradecapeptide Substrate by Pure Human Renin—The IgGs purified from peptide 81-90 antiserum were studied in further detail. After overnight preincubation at 4°C, they were tested for their ability to inhibit 0.3 mg of pure human renin acting on 5 μg of human tetradecapeptide substrate (28).

Results

Binding of Synthetic Peptides to Renin Antiserum—Twelve to 15 peptides were postulated to be epitopes of human renin according to the criteria described under “Materials and Methods,” and seven of them were synthesized. Fig. 2 illustrates the postulated three-dimensional structure of the backbone of mouse submandibular renin.

All seven peptides were tested by both RIA and ELISA for their ability to bind human renin antibodies. None of them bound to any of the monoclonal antibodies. In RIA, the three 125I labeled peptides, 50-60, Y-63-71, and Y-118-126, bound five, three, and six of the 11 polyclonal antibodies, as shown by the dilution curves in Fig. 3; whereas the four other
peptides did not bind any of these antibodies. The most representative results for binding by ELISA and RIA are compared in Fig. 4. Peptides 81–90 and 247–255, which were negative by RIA, bound two renin antisera by ELISA.

Production of Antisera against Synthetic Peptides—Antibody titers were determined for antisera collected 10 days after the third and fourth injections of bovine serum albumin-coupled peptides. Table I shows the titers of the peptide antisera determined by RIA and by ELISA. In the assay solution used for RIA, 50–68% of the iodinated peptide was bound at a final dilution of 1:25 for five antisera. Fig. 5 shows the entire dilution curve obtained with the antiserum and the corresponding purified IgG raised against the most antigenic peptide, 81–90. To preclude possible loss of binding due to iodination, the titers of the peptide antisera were determined by an ELISA test in which plates were coated with the peptides, as described under “Materials and Methods.” The five peptide antisera which bound their tracers by RIA gave a significant A/A₀ ratio in ELISA at dilutions ranging from 1:5,000 to 1:100,000 (Table I). In contrast, both RIA and ELISA showed that peptides 162–169 and 247–255 were poorly antigenic.

Recognition of Human Renin by Peptide Antibodies—The ultimate goal of the present study was to raise antibodies against peptide segments of the human renin molecule capable of recognizing the parent molecule and, by design, of inhibiting its activity.

The results are summarized in Table II. (i) The percentage binding of [³¹]I-labeled pure human renin to the peptide antisera shows that the antisera with a low titer raised against peptides 162–169 and 247–255 bound renin poorly. The antisera raised against the five other peptides bound renin significantly, with a 15–65% range at a final dilution of 1:2.5. Peptide 81–90 antiserum still bound 20% renin at a 1:25 dilution (results not shown). (ii) Renin activity was inhibited. All five peptide antisera which bound iodinated renin inhibited its enzymatic activity at a dilution of 1:5. No inhibition was found with any of the preimmune sera at the same dilutions. In addition, antipeptide 81–90 antiserum, which was the most efficient in binding renin, was also the most active in inhibiting renin since at this dilution the proportion inhibited was 68%. At a 1:50 dilution, it still inhibited plasma renin by 25%. IgG, purified from peptide 81–90 antiserum, was tested for its ability to inhibit pure human renin in its reaction with synthetic human tetradecapeptide substrate. A dose-response curve was obtained showing 36% inhibition with 0.64 mg of IgG (Fig. 6).

**DISCUSSION**

The present study demonstrated for the first time that antisera raised against short peptides constructed according
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**FIG. 3.** Recognition of the ¹²⁵I-labeled peptides by serial dilutions of the different human renin antibodies described under "Materials and Methods."

**FIG. 4.** Comparison of the amounts of synthetic peptides bound to 11 polyclonal human renin antibodies by RIA and by ELISA. For RIA (striped bars), the percent binding represents the amount of ¹²⁵I-peptide bound by an antiserum dilution of 1:25. For ELISA (open bars), the antibody dilution was 1:500 and the results are presented as the ratio of absorbance (A/A₀) due to antibody binding to peptide and to antibody binding in the absence of peptide.

**FIG. 5.** Dilution curve of a rabbit antibody raised against peptide 81-90. The binding of ¹²⁵I-peptide was determined with serial dilutions of antiserum (●) and purified immunoglobulin (▲) at corresponding concentrations in a final volume of 0.5 ml.

**TABLE I**

Titers of peptide antisera determined by RIA and ELISA

<table>
<thead>
<tr>
<th>Peptide antiserum</th>
<th>RIA</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% binding</td>
<td>Antipeptide dilution</td>
</tr>
<tr>
<td>Anti 50-60</td>
<td>64</td>
<td>1:20,000</td>
</tr>
<tr>
<td>Anti Y-63-71</td>
<td>63</td>
<td>1:5,000</td>
</tr>
<tr>
<td>Anti 81-90</td>
<td>68</td>
<td>1:100,000</td>
</tr>
<tr>
<td>Anti Y-118-126</td>
<td>50</td>
<td>1:40,000</td>
</tr>
<tr>
<td>Anti 162-169</td>
<td>0</td>
<td>1:10</td>
</tr>
<tr>
<td>Anti 247-255</td>
<td>0</td>
<td>1:10</td>
</tr>
<tr>
<td>Anti 287-295</td>
<td>52</td>
<td>1:40,000</td>
</tr>
</tbody>
</table>

**TABLE II**

Recognition of human renin by peptide antisera

Results of both binding to human renin and inhibition of its enzymatic activity are shown for one rabbit antiserum raised against each peptide. Binding to iodinated renin was obtained with an antiserum dilution of 1:2.5, and inhibition of plasma renin activity was obtained with a 1:5 dilution of the same antiserum.

<table>
<thead>
<tr>
<th>Peptide antiserum</th>
<th>Binding of human ¹²⁵I-renin %</th>
<th>Inhibition of renin activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti 50-60</td>
<td>29</td>
<td>55</td>
</tr>
<tr>
<td>Anti Y-63-71</td>
<td>19</td>
<td>50</td>
</tr>
<tr>
<td>Anti 81-90</td>
<td>65</td>
<td>68</td>
</tr>
<tr>
<td>Anti Y-118-126</td>
<td>18</td>
<td>29</td>
</tr>
<tr>
<td>Anti 162-169</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Anti 247-255</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Anti 287-295</td>
<td>15</td>
<td>33</td>
</tr>
</tbody>
</table>
to the primary structure of potential human renin epitopes are capable of recognizing pure renin and of inhibiting part of its enzymatic activity in a dose-dependent manner.

The factors that define the antigenic determinants of a protein are not clearly established, but may include the location of the epitopes on the surface of the molecule (29), their hydrophilicity (30, 31), and their segmental mobility (32).

In order to select potential antigenic determinants for synthesis, we used a model of the three-dimensional structure of mouse submandibular renin because human renin lacks a crystal structure (9). A molecular imaging apparatus enabled us to carry out graphic computer analyses of this model. With a view of obtaining antibodies able to inhibit the enzymatic activity of human renin by steric blocking, seven peptides were selected for their location near the catalytic region, characterized by a cleft containing 2 aspartyl residues involved in the aminoacyl transfer of the hydrolytic reaction.

All seven peptides were synthesized on the basis of the homologous sequence of human renin. Thus, the peptides designated 118–126 and 247–255 are located inside the cleft; whereas peptide 81–90 represents part of the flap which lies across the cleft (9), with peptide 63–71 acting as the "hinge." Peptides 30–60, 162–169, and 287–295 protrude from the molecule. In particular, sequence 287–295 corresponds to the natural internal cleavage site of mouse submandibular renin (15); whereas in human renin, this specific sequence is not hydrolyzed and contains very different charges.

Recently, two models of the three-dimensional structure have been constructed for human renin (33, 34). Using the model of Carlson et al. (34) and an algorithm in which a spherical probe comparable in size to the antibody-binding domain is allowed to contact the surface of the renin model, Evin et al. (35) predicted 12–15 peptides as potential epitopes. One of the four peptides they synthesized (the cyclic peptide C-290–296-G) is located in the same region as peptide 287–295 studied in the present work.

The recognition of synthetic peptides by human renin antibodies was examined here in order to predict their ability to be good epitopes. Polyclonal antibodies only recognized three peptides by RIA, but five by ELISA. This discrepancy might result from the incorporation into the peptides of L23I, which probably modifies conformation and subsequent binding to renin antibodies in the RIA; whereas in ELISA, unlabeled peptides were used. Sometimes, however, cross-reactivity is found with the ELISA but not with the RIA, as for peptide 50–60 with R14 and peptide 118–126 with R21 (Fig. 4). The reason for this is probably that the peptide binds the solid phase in such way that part of its antibody-binding site is inaccessible (36, 37), whereas it is accessible in the liquid phase. So the two tests were complementary. Moreover, the results of these antibody screening tests show that the 11 renin polyclonal antisera which all bound the renin molecule were different in their clonal composition. On the other hand, none of the seven peptides was recognized by the monoclonal antibodies, as previously reported (35). This is not surprising because each monoclonal antibody is directed against a single natural epitope. Moreover, attempts to locate protein epitopes by using monoclonal antibodies directed against the native molecule have led mainly to the identification of discontinuous epitopes (38, 39); whereas in the present work, the sequences of all the peptides selected were continuous. Thus, recognition of the peptides by antirenin poly- or monoclonal antibodies does not necessarily seem to be a good predictive test. Furthermore, several recent reports (29, 40, 41) show that synthetic peptides corresponding to parts of proteins are able to elicit antibodies that react with the intact antigen.

The titers of five of the peptide antisera, raised in rabbits against the seven selected peptides, ranged from 1:25 to 1:250 when measured by their binding to 125I-peptides. Similar titers have been reported for antibodies to other antigenic peptides (42). ELISA proved, however, to be a more sensitive test in our study because positive signals were obtained for dilutions ranging from 1:5,000 to 1:100,000 with the same five antisera. The two antisera directed against peptides 162–169 and 247–255 had very low titers by both RIA and ELISA. Possibly these two sequences are not immunogenic or are rapidly metabolized during the process of immunization.

The antibodies with the highest titers recognized the human renin molecule, as judged by two criteria; they both bound labeled renin and inhibited its enzymatic activity in plasma at relatively low dilutions of antisera. The results obtained in a pure system with purified antipeptide 81–90 IgG strongly suggested that antibodies raised against this part of human renin were able to bind this sequence in the whole renin molecule and to inhibit its enzymatic activity in a dose-dependent manner. It is possible that the large quantity of antibodies necessary to obtain this result is due to the great flexibility of the peptide antigen, which would imply the production of a polyclonal antiserum consisting of a mixture of monoclonal antibodies directed against a large number of conformers. Only some of these antibodies would be recognized by the same sequence, which would be more rigid in the intact renin molecule. However, Westhof et al. (32) have shown that the intact epitope in a protein possesses a relative mobility which may facilitate its adjustment to an antibody site which has not been fashioned to fit the exact geometry of a particular protein. This view may explain why in the present experiment peptide antibodies recognized renin, even at a low titer.

In conclusion, several of the peptides described here that mimic a part of the human renin sequence, especially peptide 81–90 situated in a particularly strategic region in relation to the active site, were able to produce antibodies which bound the native renin molecule and inhibited its enzymatic activity. As such, they constitute the basis for the development of a synthetic antirenin vaccine able to inhibit specifically the renin-angiotensin system.

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REFERENCES

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46. Menard, J., and Catt, K. J. (1972) *Endocrinology* 90, 422–430


