Purification of Hog Kidney Renin with Immobilized Monoclonal Antirenin

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SUMMARY Spleen cells from mice immunized with partially purified hog kidney renin were fused with mouse myeloma cells to produce a stable monoclonal hybridoma cell line that synthesizes an antibody against renin. A single monoclonal antibody was chosen for study and has been produced in large quantity and purified by affinity chromatography on protein A-Sepharose. The antirenin, which belongs to the IgG₁ subclass, exhibits anticytolytic activity against both hog and rabbit renin. An immunoaffinity column prepared from antibody coupled to Sepharose has been used in the purification of renin from hog kidney. Although renin is quantitatively adsorbed from solution, it can be eluted from the column under gentle conditions. The highly purified renin, with specific activity of 2122 Goldblatt Units/mg protein, exhibits both charge (pH 4.1 to 5.1) and size (38,000 to 42,700) heterogeneity. Hog kidney renin dissociates in the presence of sodium dodecyl sulfate (SDS) and mercaptoethanol to heavy and light chains with molecular weights of 33,700 and 5,800, respectively. In the presence of SDS, a small amount of a new form of renin is observed with a molecular weight of 19,500 which retains activity on renaturation. The monoclonal antibody should be a useful tool for the study of the renin-angiotensin system and especially for the purification of renin. The hybridoma cell line used in this study (F-32 VIII C4) has been donated to the American Type Culture Collection.

(KEY WORDS • hybridoma • immunoaffinity chromatography • angiotensin)

ANTIBODIES to renin have been an important tool in the study of renal hypertension and the renin-angiotensin system since the pioneering work of Wakerlin and Haas and Goldblatt. Until recently, antibodies to renin were nonspecific (made with impure renin as the antigen) as well as polyclonal (directed against multiple sites on the antigen). The first of these disadvantages was overcome by Dzau et al., who produced antisemur specific for dog renal renin. Both of these problems can be circumvented by the monoclonal antibody technique. First, the monoclonal antibody is specific for renin, even when an impure renin is used as the antigen, and, second, the antibody is directed against a single epitope (antigenic site) on the renin molecule. Thus, this technique allows the production of large quantities of a homogeneous antibody with defined specificity. Such antibodies should find wide use in the purification, localization, and assay of renin.

We have carried out the fusion of splenic lymphocytes from mice, immunized with partially purified hog kidney renin, with mouse myeloma cells. In this way we have established a stable monoclonal hybridoma cell line that synthesizes an antibody against renin. We describe here the production and purification of this monoclonal antirenin and its use in the purification of renin from hog kidney. The purification of renin is simple, provides a high yield of the enzyme, and, because of the unique specificity of the monoclonal antibody, gives a product of high purity.

METHODS

Renin Assay

Renin was measured in terms of the amount of angiotensin I (AI) liberated at pH 7.4 from an excess of partially purified, angiotensinase-free hog renin substrate. The bioassay of AI was performed in the anesthetized rat, and the results are expressed as pmol AI.

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The hybridoma cell line used in this study has been donated to American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852.

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formed per minute, or in terms of Goldblatt Units (GU), based on standard hog renin preparation No. 59, kindly supplied by Dr. Erwin Haas (9.1 GU/mg, Step 5 of reference 8). A GU of renin is defined as that amount of the enzyme that is required to raise the blood pressure of a trained, unanesthetized dog 30 mm Hg. and, in our assay, 1 GU is equal to 333 pmol of AI formed per minute.

**Antirenin (Anticatalytic) Assay**

For antirenin assay, 1 ml (0.02 GU) of partially purified, angiotensinase-free kidney renin (rabbit or hog) was incubated with varying amounts (1 to 50 µl) of monoclonal antirenin for 15 minutes at 37°C at pH 7.4. Then 1 ml of partially purified, angiotensinase-free hog renin substrate was added, and the incubation was continued for 30 minutes more. The reaction was stopped by boiling for 10 minutes at pH 5.5, and the AI formed was measured by bioassay, as described above. Control tubes (0.01 and 0.02 GU renin) without added antirenin were included. The amount of antirenin required to neutralize one-half (0.01 GU) of the renin was determined graphically. Antirenin concentration is expressed in terms of the amount of renin neutralized. Thus, 1 unit of antirenin is that amount that neutralizes 50% of 2 GU of renin. The rabbit and hog renin used in the antirenin assays were prepared in this laboratory and were standardized against standard rabbit and hog renin kindly supplied by Dr. Erwin Haas.

**Immunization of Mice**

The partially purified renin used as the antigen was prepared from hog kidney cortex by extraction with water, acidification to pH 2.5, precipitation with ammonium sulfate, precipitation with acetone at 50%, and chromatography on columns of DEAE-cellulose (with a NaCl gradient) and pepstatin-aminohexyl-Sepharose. The renin, with a specific activity of 346 GU per mg protein, was polymerized with glutaraldehyde. BALB/c mice received an initial subcutaneous dose of 180 µg renin with Freund's complete adjuvant. This was followed 2 weeks later by a subcutaneous dose of 360 µg renin with Freund's incomplete adjuvant. After 53 days, the mice received a second subcutaneous dose of 180 µg renin with Freund's incomplete adjuvant. This was followed 2 weeks later by an intraperitoneal dose of 360 µg of renin without adjuvant.

**Cell Fusion and Maintenance of Hybridoma Cell Lines**

Three days after the final immunizing dose, the spleens of three mice were removed, and a single cell lymphocyte preparation was made that contained about 2 × 10⁶ cells. These cells were fused with one-fifth of their number of SP2/0 Ag 14 myeloma cells, taken in the log growth phase, in the presence of 35% polyethylene glycol-1000 (Sigma Chemical Company, St. Louis, Missouri) at 37°C. After dilution in 100 ml of culture medium, the cells were then plated out in 16 96-well microtiter plates and incubated in 5% CO₂-air. The culture medium was composed of equal parts of Dulbecco's Modified Eagle Medium (Gibco, Chagrin Falls, Ohio) and RPMI 1640 (Gibco) containing 20% fetal calf serum (Gibco), 4 mM glutamine, 100 µg/ml streptomycin, 100 units/ml penicillin, and 5 g/liter glucose, and had a pH of 7.4. During the next 3 days, the medium was changed to hypoxanthine-aminopterin-thymidine-containing medium, which prevents growth of unfused myeloma cells. Every 3rd day thereafter, until the plates were discarded, the cells were fed with medium containing hypoxanthine and thymidine, but not aminopterin. During this time, aliquots of supernatant from wells that contained viable clones of cells were screened for the presence of antibodies. As soon as a clone producing antibody of interest was detected, the culture was removed from the original plate and expanded as rapidly as possible. Aliquots of 2 × 10⁶ cells were slowly frozen in phosphate-buffered saline (Flow Laboratories) that contained 10% dimethylsulfoxide and 20% fetal calf serum, and were stored in liquid nitrogen.

**Method for Screening Clones**

The radioassay used to screen for the production of antibodies was carried out as follows. In a 500 µl plastic tube were mixed 100 µl (20,000 cpm) 125I-labeled immunizing antigen and 40 µl cell culture supernatant. After incubating at 37°C for 60 minutes, 20 µl of rabbit antismouse IgG (slight excess) was added, and the mixture was incubated at 37°C for 30 minutes. Thirty µl of a 2% suspension of protein A-Sepharose CL-4B (Pharmacia, Piscataway, New Jersey) was added, and the insoluble complex was centrifuged, washed once with 100 mM NaCl/25 mM NaPO₄/pH 7.2 (PBS), and counted. In the case of clones that tested positive by the radioassay, aliquots of the cell culture supernatant were tested for antirenin by the anticatalytic assay.

**Preparation of Gamma Globulin-Free Rabbit Serum**

Commercial rabbit serum (5 liters) from Pel Freez was adjusted to pH 6.0 with 1 M HCl and fractionated with ammonium sulfate between the limits of 2.0 and 3.9 M. The resulting precipitate was dissolved in and exhaustively dialyzed against distilled water, and insoluble material was removed by centrifugation. The protein solution was diluted to the original serum volume with distilled water, adjusted to 150 mM NaCl and pH 7.2, and sterilized by filtration.

**Production and Purification of Monoclonal Antirenin**

The culture was rapidly expanded to 50 100-ml petri plates in the culture medium containing hypoxanthine and thymidine described previously. The cells were collected by centrifugation at 900 g and were transferred to a 2.5 liter spinner bottle containing 1 liter of medium that was identical except that the fetal calf serum was replaced with 10% gamma globulin-free rabbit serum that had been prepared in this laboratory. After vigorous growth had developed and the cell count had reached 1.0 to 1.5 × 10⁶ cells/ml, an additional 1.5 liters of fresh medium was added. When the concentration of cells in the spinner flask had reached...
2.5 to 3.0 x 10^6 cells/ml and cell growth had stopped (7 to 10 days), cells were removed by centrifugation.

The protein was recovered from the cell supernatant by precipitation at pH 6.0 with ammonium sulfate at a concentration of 2.0 M and was redissolved in and dialyzed against 100 mM sodium phosphate buffer, pH 8.0. The dialyzed crude antibody solution was pumped onto a 2 x 15 cm column of protein A-Sepharose CL-4B, which had been equilibrated with the same buffer. The antirenin was eluted from the column with a gradient from 100 mM sodium phosphate buffer, pH 8.0, to 50 mM citric acid, dialyzed against PBS, and concentrated by ultrafiltration.

Preparation of Affinity Adsorbents

Purified monoclonal antirenin IgG (36 mg) was coupled to 4 g of CNBr-activated Sepharose 4B (Pharmacia) at pH 8.1 according to the manufacturer's instructions. Pepstatin (Peninsula, Belmont, California) was coupled to aminohexyl-Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

Purification of Renin

Frozen hog kidney cortex (5 kg) was partially thawed and passed through a meat grinder into 10 liters of cold distilled water containing 5 mM EDTA, 2 mM phenylmethanesulfonyl fluoride, and 5 mM sodium tetraphionate. The mixture was stirred for 30 minutes, strained through gauze, and one-fifth of the volume of cold toluene was added. After stirring for 10 minutes, the mixture was centrifuged for 1 hour at 2000 g. The middle (aqueous) layer was removed by aspiration, cooled to exactly 0°C, and adjusted to pH 1.6 with 2 M HSO_4. After stirring an additional 10 minutes, the pH was adjusted to 6.2 with 5 M NaOH, and the insoluble material was removed by filtration. The pH was adjusted to 4.0, and the proteins precipitating with 2.75 M ammonium sulfate were collected by centrifugation and dialyzed against distilled water. The precipitate that formed on dialysis was discarded. This crude renin preparation was adjusted to 100 mM sodium acetate, pH 5.4, and pumped onto a 2.5 x 35 cm column of pepstatin-aminohexyl-Sepharose 4B that had been equilibrated to the same conditions. Renin was eluted from the column with a gradient from 2 M NaCl to 100 mM acetic acid. The fractions containing renin were adjusted to pH 5.0, pooled, concentrated by ultrafiltration, and washed on the ultrafilter with PBS. A pool of four such runs from the pepstatin-aminohexyl-Sepharose 4B column was pumped onto a 0.9 x 9 cm column of monoclonal antirenin-Sepharose 4B that had been equilibrated with PBS. After washing the column with the same buffer, the renin was eluted with 1.3 M NaCl/25 mM NaPO_4/pH 7.0. The tubes containing renin were pooled, concentrated by ultrafiltration, and washed on the ultrafilter with PBS. In the final step, the renin was pumped onto a 1.0 x 118 cm column of Sephacryl S-200 (Pharmacia), which was developed with PBS. The fractions containing renin were pooled and concentrated by ultrafiltration. All steps were carried out in the cold room.

Gel Filtration of Renin in the Presence of Sodium Dodecyl Sulfate

Purified renin (0.5 to 2.0 mg) was adjusted to 0.3% sodium dodecyl sulfate (SDS) by adding solid SDS at room temperature. The sample was applied to a 1.0 x 120 cm column of Sephacyl S-200 that had been equilibrated with 0.3% SDS in PBS. Fractions corresponding to protein peaks were pooled, dialyzed against distilled water, and evaporated to dryness. Complete removal of SDS was achieved by extraction with solvent system A of Henderson et al. The dried protein was dissolved in 4 M urea in PBS, added drop-wise to a 10-fold excess of PBS, and dialyzed against PBS to remove urea.

Preparation of 14C-Labeled Renin

Purified renin (500 mg, 1006 GU) was treated with a 119-fold molar excess of (1-14C-acetyl)diazooacetyl norleucine methyl ester. The pH was adjusted to 4.9, then 500 nmol cupric acetate was added, and the mixture was incubated for 3 hours. The reaction was stopped by adding 5 /mol EDTA, and the pH was adjusted to 7.0; there remained 52 GU of renin activity.

Miscellaneous Methods

Protein concentrations were determined by an automated modification of the method of Lowry et al. by using crystalline bovine serum albumin (Sigma) for standardization. Immunodiffusion was carried out by...
the Ouchterlony double diffusion method\textsuperscript{18} with goat antirabbit and rabbit antimouse antibodies (Miles, Naperville, Illinois). Concentration of dilute protein solutions was carried out by ultrafiltration with Amicon pressure cells with PM-10 membranes. Analytical disc gel electrophoresis\textsuperscript{19} was performed with a Canalco apparatus at room temperature by using 12\% gels at pH 9.5. SDS-polyacrylamide gel electrophoresis was carried out according to a modification of the method of Weber and Osborn\textsuperscript{20} by using 12\% or 15\% gels. All gels were stained with Coomassie blue R-250. Flat-bed electrofocusing was performed on an LKB model 2117 Multiphore apparatus (LKB, Gaithersburg, Maryland) by using Ultrodex gel and Ampholine solution at pH 4–6, according to the manufacturer's instructions, except that the gel bed width was reduced from 10.5 to 2.0 cm to accommodate the small samples. Sample load ranged from 1.8 to 4.6 mg protein. Separated proteins were detected by the paper print method with the use of Coomassie blue R-250. The sections of gel corresponding to stained bands were cut out, transferred to small columns, eluted with PBS, and the protein in the eluates was washed with distilled water in Amicon ultrafilters to remove ampholytes. Protein concentration in each fraction was determined by the dye-binding method of Bradford.\textsuperscript{21}

Results

Isolation of Hybridoma Cell Lines

Immunizing with renin preparations at different stages of purity and using a variety of immunization schedules, we have carried out 24 fusions. Only four of these fusions yielded hybridoma cell lines that produced antibody against the immunizing antigen. Altogether, we have isolated nine antirenin-producing clones as detected by the screening assay with \textsuperscript{125}I-labeled renin, and, of these, only three produced antibody that was anticatalytic. The hybridoma cell line (F-32 VIII C4) was chosen for study because it is stable, grows rapidly, produces an antibody that binds renin very efficiently, and exhibits anticatalytic activity.

Purification and Properties of Monoclonal Antirenin

The purification of the monoclonal antibody by affinity chromatography on a column of protein A-Sepharose is shown in Figure 1. As determined by immuno-diffusion, Tubes 3 to 9 and Tube 58 contained rabbit immunoglobulin, but no mouse immunoglobulin. Tubes 45 to 51, on the other hand, contained only mouse IgG\textsubscript{1}. The material in Tubes 45 to 51 was pooled and concentrated. It was found that 2500 ml of cell culture supernatant yielded 38 mg of purified mouse IgG\textsubscript{1}, in a subsequent large-scale preparation, 15 liters of cell culture supernatant yielded 264 mg of purified mouse IgG\textsubscript{1}. Gel electrophoresis of the purified monoclonal antirenin in the presence of SDS and mercaptoethanol showed only two bands, corresponding to heavy and light immunoglobulin chains. The purified monoclonal antirenin inhibited the catalytic activity of both hog and rabbit renin (the only renins tested with this antibody) in vitro, as shown in Figure 2. The potency of the antirenin against hog and rabbit renin was not very different: 20 units/mg protein against hog renin and 35 units/mg protein against rabbit renin, as calculated from the data in Figure 2.

Purification of Renin

The steps used to purify renin from hog kidney are shown in Table 1. The pepstatin-Sepharose affinity column was very effective and resulted in a purification of 131-fold, with recovery of 63\% of the renin activity. The use of 2 M NaCl to wash the column before eluting the renin removed nonrenin protein and was necessary to achieve this degree of purification. The next purification step, immunoaffinity chromatography on a column of monoclonal antirenin-Sepharose, is shown in Figure 3. The material in Tubes 22 to 30 was pooled, concentrated, and washed with PBS on the Amicon ultrafilter. In this step, a purification of 21-fold was achieved with 70\% recovery of renin activity. Concentrations of NaCl as low as 1 M can be used to elute the renin, although in this case the renin was eluted more slowly. On the other hand, renin can be eluted from the column in a small volume (5–10 ml) by using 3 M KSCN as the eluant. However, elution with KSCN resulted in more background material vis-

\begin{table}[h]
\centering
\caption{Purification of Renin from 20 kg of Hog Kidney Cortex}
\begin{tabular}{|l|c|c|c|}
\hline
Purification step & Protein (mg) & Renin activity (GU) & Specific activity (GU/mg) \\
\hline
2.75 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} & 29,530 & 21,904 & 0.74 \\
pepstatin-Sepharose & 141 & 13,691 & 97 \\
antibody-Sepharose & 4.7 & 9,584 & 2039 \\
Sephacryl S-200 & 4.1 & 8,702 & 2122 \\
\hline
\end{tabular}
\end{table}
Purification of hog kidney renin on a column of monoclonal antirenin-Sepharose 4B. The column was eluted with 1.3 M NaCl/25 mM sodium phosphate pH 7.0. Protein, O-O; renin activity, △-△.

Properties of Purified Renin

The purified renin obtained from the Sephacryl S-200 column appeared as one wide band in SDS gel electrophoresis with 12% gels. The molecular weight calculated from the middle of the band was 39,000 (Table 2). When the 12%-SDS gels were run in the presence of mercaptoethanol, the molecular weight was 33,700. In 15% (but not 12%) gels run in the presence of mercaptoethanol, both heavy (molecular weight 34,200) and light (molecular weight < 6500) chains were observed (Figure 5, right gel). A more accurate value for the molecular weight of the light chain could not be determined from these gels. However, the value of 5800 for the light chain was calculated by difference (Table 2). When the gels were overloaded, a faint narrow band was visible with a molecular weight of 21,500 in the absence of mercaptoethanol (Figure 5, center gel). The faint band was still present, although barely visible, with a molecular weight of 14,500 in the presence of mercaptoethanol.
TABLE 2. Molecular Weight Determinations

<table>
<thead>
<tr>
<th></th>
<th>SDS</th>
<th>SDS and mercaptoethanol</th>
<th>Difference</th>
<th>Gel filtration SDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renin</td>
<td>39,500</td>
<td>33,700</td>
<td>5800</td>
<td>43,000</td>
</tr>
<tr>
<td>Band a</td>
<td>38,800</td>
<td>33,700</td>
<td>5100</td>
<td>-</td>
</tr>
<tr>
<td>Band b</td>
<td>42,700</td>
<td>36,200</td>
<td>6500</td>
<td>-</td>
</tr>
<tr>
<td>LMR</td>
<td>21,500</td>
<td>14,500</td>
<td>7000</td>
<td>19,500</td>
</tr>
</tbody>
</table>

Gel electrophoresis was carried out on 12% polyacrylamide gels; gel filtration was carried out on a 1.0 × 120 cm column of Sephacryl S-200. Difference is the decrease in molecular weight observed when mercaptoethanol is used. Bands a and b are from renin fraction C (Figure 8) taken from a preparative electrofocusing experiment. Low molecular weight renin (LMR) is the new form of renin observed when purified renin is exposed to SDS (Figure 6, peak B).

This faint band is probably identical to the lower molecular weight peak seen when purified renin was run on the Sephacryl S-200 column in the presence of SDS (Figure 6, Peak B). The SDS-sephacryl S-200 column has been run without boiling the sample and also by adding the renin dropwise to boiling SDS buffer. In each case, the pattern obtained was the same, with about 10% of the recovered protein found in the B peak. In a separate experiment, 8856 GU of purified renin was run on the SDS-sephacryl S-200 column (sample not boiled), and the proteins in the two peaks were renatured\(^\text{13, 14}\) and assayed for renin activity. Peak A was inactive, but Peak B had 89 GU with a specific activity of 342 GU/mg protein. When purified renin was reacted with the active site-directed reagent (\(^1\)\(^4\)-C-acetyl)diazoacetyl norleucine methyl ester and run on the Sephacryl S-200 column in the presence of SDS, radioactivity coincided with protein in both Peaks A and B.

When purified renin was examined under native conditions by disc gel electrophoresis, three very close bands were seen (Figure 5, left gel). An unstained gel was cut into 1 to 2 mm sections, and each was eluted and assayed for renin activity. Renin was found in all of the sections corresponding to the stained bands. We have previously observed that when partially purified hog kidney renin is subjected to preparative vertical slab gel electrophoresis, three electrophoretically distinct forms of renin with similar specific activity can be isolated (Lentz, Skeggs, Dorer, Kahn, Levine, unpublished data). These probably are multiple forms of renin differing in isoelectric point.\(^\text{6-23}\) The purified renin was subjected to preparative electrofocusing over the pH range 4 to 6. In four such experiments, renin activity focused over the pH range 4.1 to 5.1. A typical paper print pattern is shown in Figure 7. From

![Figure 6](http://hyper.ahajournals.org/)

**FIGURE 6.** Gel filtration of purified hog kidney renin on a column of Sephacryl S-200 in the presence of 0.3% SDS. Solid SDS was dissolved in the sample which contained 0.5 mg protein just before applying the sample to the column. The column was developed with 0.3% SDS in PBS at room temperature.

![Figure 7](http://hyper.ahajournals.org/)

**FIGURE 7.** Preparative flat-bed electrofocusing of purified hog kidney renin with pH 4–6 Ampholine solution. The sample contained 3.0 mg protein. Separated protein bands are visualized by the paper print method using Coomassie blue R-250.
one of these experiments, eight fractions were isolated with isoelectric points from 4.15 to 4.85. All fractions had renin activity with specific activities ranging from 1807 to 2684 GU/mg protein. Six of these fractions were examined by SDS gel electrophoresis (Figure 8). Fractions B, C, and D show two bands differing in molecular weight by 3900. The presence of renins with small differences in molecular weight probably explains the single wide band that is observed when purified renin is subjected to SDS gel electrophoresis.

To show that the multiple forms of renin were not formed as a result of the pH 1.6 step of the purification process, we have purified renin from 30 kg of hog kidney cortex by the procedure described above, but have replaced the pH 1.6 step with a batch DEAE-cellulose step.6 The renin obtained in this way had a similar specific activity (2359 GU/mg protein), although the yield was lower (3.12 mg protein). This renin was indistinguishable from the acid-treated renin in SDS gel electrophoresis, both with and without mercaptoethanol: by electrofocusing that showed renin activity over the pH range of 4.45 to 5.12; and by gel filtration on Sephacryl S-200 in the presence of SDS after treatment of the renin with (1-14C-acetyl)diazolec-acyl norleucine methyl ester.

Purified hog kidney renin (3.5 mg, 6650 GU) was treated with 6 M urea (Schwarz/Mann “ultra-pure”) for 50 hours at 5°C. Following dialysis to remove urea, the sample was run on the monoclonal antirenin-Sepharose column. The protein was not adsorbed, and the column effluent contained 2.9 mg protein and 2 GU renin. The 1.3 M NaCl eluate contained no detectable protein and 9 GU renin. This would suggest that the antigenic site is conformational rather than sequential.

**Figure 8.** SDS gel electrophoresis (12% polyacrylamide gel, no mercaptoethanol) of purified renin fractions isolated by preparative electrofocusing. Because only small amounts of some of the fractions were available, no attempt was made to apply an equivalent amount of each fraction or a constant amount of protein to each gel. The pH values at which each fraction focused, and the specific activity of each fraction, are given.

**Discussion**

Use of antirenin as a tool to study the role of the renin-angiotensin system in the regulation of blood pressure has yielded equivocal results because of the nonspecificity of the antibodies that have been available. Recently, Dzau et al.3 have produced renin-specific antibody by using purified dog renal renin and have used this antibody to reduce blood pressure in dogs with acute renovascular hypertension.

The development of the monoclonal antibody technique in recent years has allowed the production of theoretically unlimited amounts of antibody with defined specificity. The monoclonal hybridoma cell line, which we have isolated, is stable and produces an antibody directed against a single epitope in the renin molecule. This antigenic site must be near the catalytic site, since enzyme activity is inhibited. That the antibody is not directed at the catalytic site is inferred from the observation that the anticatalytic activity calculated from the data in Figure 2 is only 20 units per mg antibody protein, while the capacity of the antirenin column to bind hog renin is greater than 300 GU per mg antibody protein. It is surprising that the anticatalytic activity against rabbit renin is greater than against hog renin (Figure 2). However, this might be explained by the fact that the antirenin assays were carried out using equipressor (GU) rather than equimolar amounts of enzyme. In addition, the antigenic site must be conformational rather than sequential, since urea-denatured renin loses its ability to bind the antibody. Dzau et al.28 have isolated several monoclonal antibodies to dog renal renin. One of these antibodies cross-reacts with renin from human, hog, bovine, and mouse kidney. Interestingly, none of the antibodies exhibited anticatalytic activity. On the other hand, Pau et al.27 and Simon et al.28 have isolated a monoclonal antibody against human tumor renin that does possess anticatalytic activity. This antibody reacts with both human and monkey renin, but does not recognize hog or mouse renin.

We originally attempted to detect monoclonal antibodies directed against renin with an ELISA (enzyme-linked immunoassorbent assay)29 by using renin bound to polystyrene microtiter plates. However, the ELISA proved to be unsatisfactory, and the radioassay with 125I-labeled renin was developed. The purification of the monoclonal antirenin in large quantities from the culture medium has been aided by our use of rabbit serum, from which nearly all of the gamma globulin has been removed by fractionation with ammonium sulfate, in place of bovine serum. The small amount of rabbit gamma globulin that is present in the culture
Renin has been purified from hog kidney cortex using affinity chromatography in combination with other chromatographic and electrophoretic techniques. Specific activities as high as 2000 GU/mg protein and yields of 0.1 mg renin/kg kidney tissue have been reported. However, the procedure described here is the first reported use of monoclonal antirenin in the large-scale purification of kidney renin. The use of the antirenin-Sepharose column along with the peptatin-aminohexyl-Sepharose chromatography step has permitted a rapid and efficient purification of renin with a specific activity of at least 2000 GU/mg protein and recovery of 0.2 mg renin/kg kidney cortex. It is possible that the crude extract could be applied directly to the monoclonal antirenin-Sepharose column and could eliminate the need for the peptatin affinity chromatography step. However, since we process such large amounts of kidney tissue, we feel that the procedure we have developed will prolong the useful life of the monoclonal antirenin-Sepharose and will give a better final product. The low affinity of the antirenin-Sepharose for renin is a very important factor, since it allows the renin to be eluted under mild conditions. The monoclonal antirenin-Sepharose prepared by Pau et al. also exhibited low affinity for renin (human amniotic fluid and tumor renin) which could be eluted by lowering the pH to 4.5.

Despite the use of enzyme inhibitors introduced by Murkami and Inagami, the renin obtained by our purification procedure must consist of a family of proteins with isoelectric points ranging from 4.1 to 5.1. The fact that we can see this degree of heterogeneity is due at least in part to the very high resolving power of the flat-bed electrofocusing technique. Charge heterogeneity of hog kidney renin has been reported before, although the renin isolated by Inagami and Murakami showed only a single band at pH 5.2 on polyacrylamide gel electrophoresis. This may have been due to the fact that they took a rather narrow cut on the DEAE-cellulose chromatography step during their purification procedure. The charge heterogeneity observed by us is not necessarily a result of the treatment at pH 1.6 in our purification procedure, since we have also purified renin with the use of a batch DEAE-cellulose step at a more neutral pH. In this case, electrofocusing of the purified renin showed renin activity over the pH range 4.45 to 5.12. The charge heterogeneity is no doubt similar to that observed several years ago in this laboratory with partially purified hog kidney renin that had been exposed to a pH no lower than 4.6 during the purification procedure. The purified renin also exhibits some size heterogeneity, which is evident in SDS gel electrophoresis (Figures 5 and 8), but is not seen by gel filtration (Figure 4). For example, Fraction B in Figure 8 shows two clearly separated bands differing in molecular weight by 3900.

The new low molecular weight (LMR) form of renin, which is observed when renin is run on the Sephacryl S-200 column in the presence of SDS (Figure 6, Peak B), is also seen as a very faint band in SDS gel electrophoresis when the gels are overloaded (Figure 5). Since it was important for us to show that our renin preparation was free of nonrenin contaminants, we have accumulated evidence to show that this LMR really is a form of renin. First, LMR was obtained from protein fractions that had adsorbed to both immobilized pepstatin and antirenin. Second, we have shown that LMR possesses renin catalytic activity after removal of SDS and renaturation. Third, we have shown that the active site reagent, diazooctyl norleucine-methyl ester, is incorporated into LMR. Fourth, LMR seen on SDS gel electrophoresis changes molecular weight from 21,500 to 14,500 when treated with mercaptoethanol (Table 2).

In addition, we have observed LMR in the renin prepared with the use of the batch DEAE-cellulose step in place of the pH 1.6 step, as described above. LMR may arise from the two halves of a renin molecule that have been "cut" by a proteolytic enzyme in an accessible, susceptible region at some stage during post-translational processing or during the extraction and purification procedure. In view of the fact that Peak B (Figure 6) always represents 10% to 12% of the recovered protein after SDS treatment (we have run this column 11 times), random formation during the extraction and purification procedure seems less likely. After this proteolytic cleavage, the two domains of renin could have been held together by noncovalent forces, coeluted with intact renin from the Sephacryl S-200 column in the absence of SDS (Figure 4), and then been dissociated by mild treatment with SDS, by appearing at 19,500 molecular weight when renin on the Sephacryl S-200 column in the presence of SDS (Figure 6, Peak B). Galen et al. have observed the presence of human kidney tumor renins with molecular weight 20,000 and 25,000 by gel electrophoresis under dissociating conditions. These authors were unable to detect these smaller renins under nondenaturing conditions.

As shown in Table 2, we have found the molecular weight for purified hog kidney renin to be 39,500 by SDS gel electrophoresis under nonreducing conditions, and 43,000 by gel filtration in the presence of SDS. Of the two, we are more confident of the value of 39,500 since we have performed the molecular weight determination by SDS gel electrophoresis several times with good agreement. When renin was examined by SDS gel electrophoresis under reducing conditions with 15% gels, we observed two bands, one at a molecular weight of 34,200 and the other at a molecular weight estimated at less than 6500. Since mouse submaxillary gland renin has been shown to contain heavy and light chains linked by one disulfide bridge, we speculate that what we have observed are the corresponding peptide chains in hog kidney renin. Thus, a calculated value of 5800 was obtained for the molecular weight of the light chain (Table 2). Inagami and
Murakami\(^{22}\) obtained molecular weight values of 42,500 and 35,000 for hog kidney renin by SDS gel electrophoresis under nonreducing and reducing conditions, respectively. They predicted the existence of the light chain, although they could not detect it on their 7.5% gels. Corvol et al.,\(^{25}\) on the other hand, found the same value (36,800) for the molecular weight of hog kidney renin by SDS-gel electrophoresis under both reducing and nonreducing conditions.

Monoclonal antibodies such as the one we have described will no doubt prove to be useful tools in the study of the renin-angiotensin system and will find wide use in the identification, localization, and purification of renin.

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