Characterization of Five Epitopes of Human Renin from a Computer Model†

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ABSTRACT: Antigenic determinants in proteins have been found to coincide with surface regions accessible to a large probe of 1-nm radius, which approximates the size of an antibody binding domain. To predict epitopes of human renin, a computer algorithm was used to calculate the accessibility of a model of the three-dimensional structure of human renin to a 1-nm probe. Of the 17 segments predicted to be epitopes or parts of epitopes, 6 were synthesized and tested for their recognition by 11 polyclonal antibodies prepared against pure human renin. Four peptides, Y-133–144, C-180–188, Y-211–224, and Y-300–310, were bound by some polyclonal anti-renin antibodies when tested in both a solution assay and an enzyme-linked immunosorbent assay (ELISA), and one peptide, C-290–296-G, was bound only in an ELISA. Of the five epitopes identified, peptide Y-211–224 seems dominant, since it was bound by all antisera in the solution assay and by 8 of the 11 in the ELISA. This peptide contains a disulfide loop, and the corresponding linear peptide obtained after reduction of the disulfide was not recognized by any anti-renin antisera in the solution assay. This suggests that the epitope is conformationally dependent and that a disulfide bridge linking cysteines-217 and -221 is present in the native structure of human renin. Purified immunoglobulins raised to peptides Y-133–144 and Y-211–224 (located near, but not at, the catalytic site) were found to inhibit the cleavage of human angiotensinogen by purified human renin but not the cleavage of a tetradecapeptide substrate representing the N-terminal region of angiotensinogen. Immunoglobulin specific for peptide Y-300–310 (located at the edge of the cleft opposite the flap) inhibited cleavage of both angiotensinogen and tetradecapeptide. Immunoglobulins specific for C-180–188, a peptide more distant from the catalytic site, had no effect on enzymatic activity.

Renin (EC 3.4.23.15) is a very specific aspartyl protease that cleaves angiotensinogen to release the decapeptide angiotensin I. An octapeptide from the N-terminal end of angiotensinogen is the minimal sequence that the enzyme will cleave. The reaction of renin on angiotensinogen is an early step in the renin–angiotensin–aldosterone cascade, one of the systems responsible for the maintenance of blood pressure and of fluid and electrolyte balance (Oparil & Haber, 1974). Human renin has been purified and characterized (Galen et al., 1979a; Slater et al., 1981; Yokosawa et al., 1980), and its primary structure has been established through recombinant DNA techniques (Imai et al., 1983).

Because human renin has been available only in very limited quantities, an X-ray crystallographic study of its structure has not yet been possible. However, several models of its three-dimensional structure have been proposed on the basis of the structures of homologous proteins and renin's amino acid sequence (Akahane et al., 1985; Carlson et al., 1985; Sibanda et al., 1984). The model proposed by Carlson et al. was constructed by maximizing the sequence homology between human renin and three other aspartyl proteases whose tertiary structures have been defined by X-ray diffraction studies to a resolution of 2 Å or better and was refined and analyzed with energy minimization programs.

To evaluate the renin model proposed by Carlson et al., we sought to experimentally verify the antigenic epitopes predicted to be on its surface. Several groups (Fanning et al., 1986; Novotný et al., 1986; Thornton et al., 1986) have recently shown that antigenic epitopes coincide with the most protruding regions on protein surfaces. Our own group (Novotný et al., 1986) has used a large-probe accessibility algorithm to calculate the contacts between a 1-nm-radius probe, the approximate size of an antibody binding domain, and the three-dimensional structure of a protein as determined from crystallographic data. A good correlation has been found between surface accessibility and antigenicity when the algorithm is applied to proteins such as lysozyme or myoglobin whose X-ray crystallographic structures and antigenic determinants have both been defined. When this algorithm was applied to the model of human renin, 17 segments were predicted to be epitopes (Evin et al., 1986). We studied 6 of them by synthesizing the corresponding peptides and testing for binding by 11 polyclonal and 7 monoclonal antibodies raised against pure human renin. Peptides effectively bound by the anti-renin antibodies were thus identified as renin epitopes and used as haptens to raise polyclonal antibodies in rabbits. The antipeptide antibodies were then tested for their ability to inhibit renin enzymatic activity.

Materials and Methods

Computer Modeling and Epitope Prediction. A model of the three-dimensional structure of human renin was previously constructed in our laboratory (Carlson et al., 1985). It is based on the three-dimensional structures of three homologous aspartyl proteases that had been previously studied by X-ray diffraction at a resolution of 2.1 Å or better and refined by Konnert–Hendrickson algorithms (James & Sielecki, 1983;

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Table I: Physical Characteristics of the Synthetic Peptides

peptide identification	sequence	HPLC[a] retention (min)	amino acid analysis
Y-133–144	YIEQAIGRVTPIF	12.35	Thr 0.99 (1), Glu 2.04 (2), Pro 1.09 (1), Gly 1.05 (1), Ala 1.01 (1), Val 1.01 (1), Ile 2.80 (3), Tyr 0.87 (1), Phe 0.94 (1), Arg 1.04 (1)
Y-148–156	YISQGVLKED	8.44	Asp 1.06 (1), Ser 0.90 (1) Glu 2.18 (2), Gly 1.02 (1), Val 0.94 (1), Ile 0.87 (1), Leu 0.90 (1), Tyr 0.94 (1), Lys 1.04 (1)
C-180–188	CGSDPQHYEG	7.53	Asp 0.96 (1), Ser 0.94 (1), Glu 2.37 (2), Tyr 0.84 (1), His 0.95 (1)
Y-215–224	YLLCEDGCLAL	12.74	Asp 1.00 (1), Glu 1.05 (1), Gly 1.06 (1), Ala 1.05 (1), Leu 3.85 (4), Tyr 0.43 (1)
	YLLCEDGCLAL (Scm Scm)	11.84	Asp 0.97 (1), Glu 0.93 (1), Gly 1.09 (1), Cys(Scm) 1.71 (2), Ala 1.12 (1), Leu 4.18 (4), Tyr 0.76 (1)
Y-211–224	YGSSTLLCEDGCLAL	12.72	Asp 1.04 (1), Thr 0.96 (1), Ser 1.85 (2), Glu 1.03 (1), Gly 1.98 (2), Cys 1.69 (2), Ala 1.02 (1), Leu 4.15 (4), Tyr 0.95 (1)
	YGSSTLLCEDGCLAL (Scm Scm)	14.68	Asp 0.97 (1), Thr 0.97 (1), Ser 1.80 (2), Glu 1.05 (1), Gly 2.10 (2), Cys(Scm) 1.86 (2), Ala 1.13 (1), Leu 4.10 (4), Tyr 0.43 (1)
C-290–296-G	CYSSKKLCG	8.43	Ser 2.04 (2), Gly 1.07 (1), Leu 1.01 (1), Tyr 0.75 (1), Lys 2.10 (2)
	CYSSKKLCG (Scm Scm)		Ser 1.93 (2), Gly 0.92 (1), Cys(Scm) 2.10 (2), Leu 0.95 (1), Tyr 0.41 (1), Arg 2.20 (2)
C-287–297	CQESYSSKKLCT	8.53	Thr 0.96 (1), Ser 3.01 (3), Glu 2.02 (2), Cys 0.42 (2), Leu 1.03 (1), Tyr 0.85 (1), Lys 2.12 (2)
287–297	QESYSSKKLCT	7.95	Thr 1.02 (1), Ser 2.90 (3), Glu 2.00 (2), Cys 0.77 (1), Leu 1.08 (1), Tyr 0.65 (1), Lys 1.99 (2)
285–292	VFQESYSS	8.80	Ser 2.84 (3), Glu 2.10 (2), Val 0.99 (1), Leu 0.88 (1), Phe 1.18 (1)
[258–262]-SS-[287–297]	[KCNEG]-SS-[QESYSSKKLCT]	7.57	Asp 0.99 (1), Thr 0.95 (1), Ser 2.91 (3), Glu 3.12 (3), Gly 1.01 (1), Cys 1.55 (2), Leu 0.98 (1), Tyr 0.44 (1), Lys 3.01 (3)
Y-300–310	YIHAMDIPPPTG	9.45	Asp 1.04 (1), Thr 1.04 (1), Pro 3.17 (3), Gly 1.08 (1), Ala 1.02 (1), Met 0.99 (1), Ile 1.75 (2), Tyr 0.69 (1), His 0.87 (1)

[a] The peptides were eluted on a Vydac C-18 column (4.6 × 250 mm) at a flow rate of 2 mL/min with a gradient increased linearly over 12 min from 0 to 50% acetonitrile in water in the presence of 0.05% trifluoroacetic acid.

D. Davies and T. Blundell, personal communication). The model was then further analyzed with the energy refinement and molecular dynamics program CHARMM (Brooks et al., 1983).

The segments of a protein with the highest calculated accessibility to a 1-nm probe have been found to correlate well with the antigenic epitopes of a number of proteins whose three-dimensional structures are known through X-ray diffraction studies (Novotný et al., 1986). To determine the regions of the renin model most likely to correspond to antigenic epitopes, the static accessibility of the model to a spherical probe of 1-nm radius was calculated by modifying the algorithm developed by Lee and Richards (1971) for determining static accessibility to a spherical probe of 1.4-Å radius. As the 1-nm spherical probe is rolled across the surface of the renin model, the surface area of each atom capable of coming into contact with it is calculated in Å^2. The surface area is then summed for all atoms in a particular amino acid and divided by the surface area calculated for the same amino acid in a tripeptide with an extended-chain conformation. The accessible surface area for each amino acid in the model, expressed as a percentage, is then smoothed by a seven-point moving window procedure (Novotný et al., 1984). To determine whether there were any conformations that could be preserved in the peptides to be synthesized, the structures of each amino acid segment calculated to have high accessibility to the 1-nm probe were visualized on an Evans and Sutherland PS340 computer graphics display running the FRODO software originally developed by Jones (1978) and modified by Pflugrath et al. (1984).

Peptide Synthesis. Table I lists the characteristics of all peptides prepared for this study. The peptides were synthesized by solid-phase techniques according to Merrifield (1964, 1986). In brief, 2 g of *p*-methylbenzhydrylamine resin (purchased from U.S. Biochemicals, Cleveland, OH) was reacted with a 4-fold excess of the Boc[1] amino acid derivative (obtained from Peninsula, Belmont, CA) and (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) prepared according to Castro and Dormoy (1979) (also available from Fluka and Novabiochem) in the presence of diisopropylethylamine. For best coupling efficiency, the pH was adjusted to 7.5. The Boc protecting group was cleaved by treating the resin with 50% trifluoroacetic acid in dichloromethane (v/v). Complete side-chain deprotection and cleavage from the resin were realized by treatment with liquid hydrogen fluoride containing 10% anisole for 45 min at 0 °C. The crude peptide was extracted with dilute acetic acid and lyophilized. For disulfide bond formation by air oxidation, the crude material was dissolved in 0.02 M ammonium acetate, pH 8.7 (0.2 mg of peptide/mL), and allowed to stand for 24 h at room temperature. No free sulfhydryl could be detected by Ellman's test (Ellman, 1959), and reaction with iodoacetamide did not show any formation of (carboxymethyl)cysteine, as checked by amino acid analysis. Disulfide linkage of the two peptides KCNEG and QESYSSKKLCT was done under the same conditions with a 2.5-fold excess of the pentapeptide over the tridecapeptide. All peptides were purified by gel filtration on Sephadex G-25 followed by ion-exchange chromatography on CM-52 or DEAE-52 cellulose (depending upon the relative ionic charges of the peptide). Purity was assessed by amino

[1] Abbreviations: Boc, *N-tert*-butyloxycarbonyl; BSA, bovine serum albumin; cpm, counts per minute; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline; PBSA, phosphate-buffered saline containing 0.02% sodium azide; Scm, *S*-carboxymethyl; Tris, tris(hydroxymethyl)aminomethane; CM, carboxymethyl; DEAE, diethylaminoethyl.

acid analysis on a Dionex Model 5000C automatic analyzer and by HPLC on a Vydac C-18 column, using a Waters system equipped with a gradient monitor (Millipore–Waters Associates, Medford, MA). Reduction of the disulfides and carboxymethylation of the cysteines were carried out by allowing the disulfide peptide (10 mM in 0.01 M phosphate, pH 8.7) to react with dithiothreitol (10 mM) for 1 h at 37 °C and then with iodoacetamide (22 mM) for 15 min at 0 °C. Excess reagent was removed by filtration on Bio-Gel P-2 (200–400 mesh, Bio-Rad, Richmond, CA). The peptides were also characterized by their retention time on a reverse-phase HPLC column.

Binding Assay of the Synthetic Peptides to Anti-Renin Antibodies. The preparation of the polyclonal (Galen et al., 1979b) and monoclonal (Galen et al., 1984) anti-renin antibodies has already been described. Seven monoclonal (F15, 1A12, 2D12, 4E1, 3E8, 4B11, and 4G1) and 11 polyclonal (R11, R12, R14, R15, R21, R22, R24, R25, R26, Donc, and Bof) antibodies were available that had been prepared with pure human active renin from a kidney tumor. This tumoral renin was found to have the same biochemical, biophysical, and immunological characteristics as human renal renin (Galen et al., 1979a).

For the solution assays, the peptides were radiolabeled with sodium [^{125}I]iodide in the presence of chloramine T (Greenwood et al., 1963) or, for those containing cysteine and methionine residues, with IodoGen (Pierce Chemical Co., Rockford, IL) according to the method of Fraker and Speck (1978). Each labeled peptide was purified on a Sep-Pak C-18 cartridge (Millipore–Waters Associates, Medford, MA) and eluted with 40% acetonitrile in 1% trifluoroacetic acid in water (v/v). A tracer amount of peptide (5000 cpm) was incubated with 2.5 and 25 dilutions of antibody in phosphate buffer (0.1 M, pH 7.5) for 24 h at 4 °C. The IgG fraction was precipitated with 20% poly(ethylene glycol), as described previously (Galen et al., 1984), separated by centrifugation, and counted. Nonspecific binding (6%) determined with nonimmune rabbit serum was subtracted from the given values.

For the ELISA, poly(vinyl chloride) 96-well microtiter plates (Falcon Labware, Becton-Dickinson and Co., Oxnard, CA) were coated with 5–10 mg/mL of peptide in 0.1 M Tris buffer (pH 9.6) for 16 h. After saturation of the nonspecific binding sites with 1% bovine serum albumin (BSA, radioimmunoassay grade, obtained from Sigma) in 0.1 M phosphate-buffered saline (PBS), pH 7.4, 1:500 dilutions of antibody in PBS were incubated for 30 min at 37 °C. After extensive washing, alkaline phosphatase labeled anti-rabbit (or anti-mouse) γ-globulin (Sigma) was added and incubated for 30 min at 37 °C, followed by *p*-nitrophenyl phosphate. A colorimetric reaction developed that was quantitated by reading the absorbance (A) at 405 nm, after 1–2 h of incubation of the enzyme. Wells incubated under the same conditions but not coated with peptide provided the control values (A_0). Data were expressed as ratios A/A_0. Only ratio values greater than 2 were interpreted as significant.

Immunization. Peptides were conjugated to keyhole limpet hemocyanin (KLH) or thyroglobulin in the presence of glutaraldehyde (Reichlin, 1980). For conjugation to KLH, 0.5 mL of a 50% glycerol suspension (approximately 31 mg of KLH; purchased from Calbiochem) was diluted 4 times with 0.05 M phosphate buffer, pH 7.5. This cloudy solution was transferred to a vial containing 7–10 mg of peptide. Glutaraldehyde (200 μL of a 25% aqueous solution obtained from Sigma) was diluted with water (1 mL) and added within 15 min by 50-μL aliquots to the peptide–protein solution, with stirring. Stirring continued overnight, and the reaction mixture was dialyzed against 2 L of distilled water. An approximate value of the conjugate's peptide content was determined by amino acid analysis of an aliquot of the dialyzed solution. The yield ranged from 10 to 15 mol of peptide/100 kDa of KLH. For conjugation to thyroglobulin, 20 mg of bovine thyroglobulin (Sigma) and 7–7.5 mg of peptide were dissolved in 3 mL (0.1 M) of PBS, pH 7.4. Glutaraldehyde was diluted and gradually added, as described above. Approximately 3–3.5 mg of peptide was incorporated per 20 mg of protein, as determined by amino acid analysis.

Two male New Zealand rabbits were injected intradermally with each peptide conjugate (100–200 μg of peptide; 0.7–1.7 mg of conjugate) in the presence of complete Freund's adjuvant. The animals were boosted every 4 weeks with the same amount of conjugate, in the presence of incomplete adjuvant. After the first booster injection, blood was collected every week, except for the weeks of immunization.

Screening of Antipeptide Antisera. Antipeptide antisera were tested for their binding to the iodinated hapten in a solution assay. ^{125}I-Labeled peptides were prepared as described above. Tracer amounts of peptide (5000–7000 cpm) were incubated overnight with various dilutions of antisera, in 1% BSA/PBSA, pH 7.4. Charcoal (a suspension of 1.25 g of Norit A and 0.25 g of Dextran T-70 in 100 mL of PBSA, pH 7.4) was used to separate bound from free tracer. After centrifugation, the supernatants were decanted and counted. Nonspecific binding was 7–9%, as determined by a 1:100 dilution of preimmune serum. Titers were estimated as antibody dilutions capable of binding 50% of tracer.

The antisera were also tested in a solid-phase assay. Hapten peptide (20 μg/mL) was preadsorbed on poly(vinyl chloride) 96-well microtiter plates. Nonspecific binding sites were blocked with 1% BSA/PBSA as described above. After the plates were extensively washed, antiserum was applied (10–10 000-fold dilutions, in 1% BSA/PBSA) and the solution was incubated for 2 h at room temperature. After the plates were again washed, ^{125}I-labeled protein A (freshly prepared by the chloramine T method) was added (20 000 cpm per well) and the solution incubated for 1 h. The plates were again washed and the wells were cut and counted. A peptide not related to the immunogen was tested under the same conditions to determine the nonspecific binding. Preimmune serum (1:100 dilution) was assayed as a control.

Purification of Antipeptide Immunoglobulin G (IgG). Antipeptide antiserum (2 mL) was diluted with 0.5 M phosphate buffer, pH 8.0 (0.5 mL), and applied on a 2-mL protein A–Sepharose affinity column (Ey et al., 1978) preequilibrated with 0.1 M phosphate buffer, pH 8.0 (protein A–Sepharose CL-4B, Sigma; binding capacity approximately equal to 20 mg of human IgG/mL of swollen gel). After the column was washed with 20 mL of 0.1 M phosphate buffer, pH 8.0, the IgG were eluted with 0.1 M acetic acid. Fractions of 0.8 mL were collected and immediately neutralized by adding 0.1 mL of 1 M Tris. The fractions giving the highest absorbance values at 280 nm were pooled, dialyzed against 0.02 M PBSA (pH 7.5), and lyophilized. Before lyophilization, the IgG concentration was estimated from the dialyzed solution by using Bio-Rad protein assay reagent and a standard curve for bovine γ-globulins (Sigma).

Inhibition Assay of Human Renin Activity. Pure human renin (0.3 ng) was incubated overnight at 4 °C with 200 μL (0.5 mg) of purified antipeptide IgG in 0.1 M phosphate buffer (pH 7.5). Renin substrate was then added [either 2.5 μg of pure human angiotensinogen, obtained from Dr. D. Tewks-

Table II: Sequence and Numbering of the Renin Segments Studied as Antigenic Determinants

predicted epitope no.	peptide identification	sequence
6	Y-133–144	(Tyr)-Ile-Glu-Gln-Ala-Ile-Gly-Arg-Val-Thr-Pro-Ile-Phe-amide[a]
7	Y-148–156	(Tyr)-Ile-Ser-Gln-Gly-Val-Leu-Glu-Asp-amide
9	C-180–188	(Cys)-Gly-Ser-Asp-Pro-Gln-His-Tyr-Glu-Gly-amide
10	Y-215–224	(Tyr)-Leu-Leu-Cys-Glu-Asp-Gly-Cys-Leu-Ala-Leu-amide
	Y-211–224	(Tyr)-Gly-Ser-Ser-Thr-Leu-Leu-Cys-Glu-Asp-Gly-Cys-Leu-Ala-Leu-amide
15	C-290–296-G	(Cys)-Tyr-Ser-Ser-Lys-Lys-Leu-Cys-(Gly)-amide
	C-287–297	(Cys)-Gln-Glu-Ser-Tyr-Ser-Ser-Lys-Lys-Leu-Cys-Thr-amide
13–15	[258–262]-SS-[287–297]	Lys-Cys-Asn-Glu-Gly-amide | Gln-Glu-Ser-Tyr-Ser-Ser-Lys-Lys-Leu-Cys-Thr-amide
16	Y-300–310	(Tyr)-Ile-His-Ala-Met-Asp-Ile-Pro-Pro-Pro-Thr-Gly-amide

[a] Residues shown in parentheses are those that do not exist in the native structure of human renin.

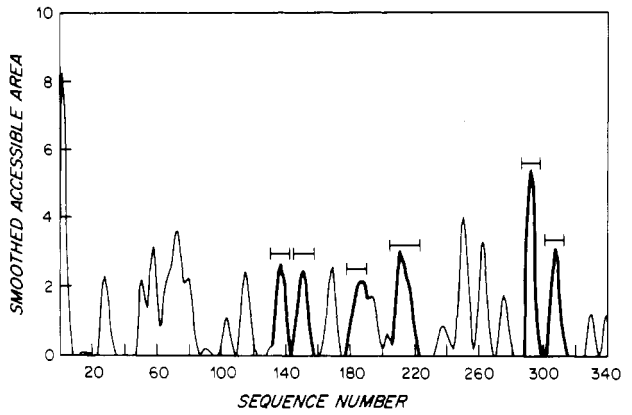

FIGURE 1: Accessibility profile of the human renin model to a 1-nm-radius probe. Bold lines and range bars indicate peptides selected for synthesis.

bury, or 5 μg of synthetic human tetradecapeptide, obtained from Dr. D. Le Nguyen, as previously reported by Cumin et al. (1985)]. The enzymatic reaction was allowed to proceed for 2 h at 37 °C in 0.1 M phosphate buffer, pH 5.7, in the presence of phenylmethanesulfonyl fluoride and was quantitated by radioimmunoassay of generated angiotensin I, as reported by Guyenne et al. (1979). Total renin activity (100%) was determined by incubating human renin under the same conditions, except that in the preincubation the IgG solution was replaced by phosphate buffer.

Results and Discussion

Many different approaches to the prediction of antigenic epitopes have been described, using either the amino acid sequence of a protein or its three-dimensional structure [for a review, see Berzofsky (1985)]. If a three-dimensional structure is available, it provides considerable advantage, since it is generally agreed that epitopes recognized by B-cell receptors or antibodies are on the surface of protein molecules. If only the amino acid sequence is available, other strategies must be employed. Hopp and Woods (1981) have utilized computed hydrophilicity parameters of the amino acids in a protein's primary structure to identify presumed hydrophilic regions. They reasoned that these are more likely to be surface exposed than hydrophobic regions and thereby define possible epitopes.

Another approach to predicting epitopes relies on the immunologic concept of tolerance. It is reasoned that, with respect to homologous proteins, those regions that are conserved among species are unlikely to be immunogenic, whereas those regions that vary are recognized as foreign and therefore become epitopes. Thus another method of predicting epitopes is to select regions of protein sequence that lack homologies

Table III: Binding of the Synthetic Peptides to 11 Anti-Renin Antisera Measured by ELISA[a]

anti-renin anti-serum	Y-133–144	Y-148–156	C-180–188	Y-215–224	Y-211–224	Y-300–310
R11	*2.7*[c]	1.1	1.4	1.6	*2.5*	*2.8*
R12	1.3	1.2	1.7	1.7	*4.5*	*6.8*
R14	1.0	1.1	1.2	1.5	*11*	1.3
R15	1.1	1.0	1.7	1.4	*3.0*	1.0
R21	1.0	0.9	1.2	1.9	*15*	1.0
R22	2.0	1.0	*2.3*	*2.4*	*8.5*	1.3
R24	1.1	1.2	*10.3*	1.5	2.0	1.1
R25	1.0	1.1	*3.9*	1.3	*7.5*	1.0
R26	1.1	1.3	*3.5*	1.8	2.0	1.1
Donc	1.2	1.0	1.4	1.6	*7.0*	1.1
Bof	1.4	ND[b]	1.5	1.5	1.7	1.0

[a] Results are presented as the ratio of absorbance (A/A_0) due to antibody (1:500 dilution) binding to peptide and antibody binding in the absence of peptide. [b] ND, not determined; no more antiserum available. [c] Data shown in italics were those interpreted as significant binding of peptide to antiserum.

in other proteins, as determined from protein data banks (Delmas et al., 1985), or to select specific regions of nonhomology in otherwise closely homologous proteins (Krystek et al., 1985). Because most sequence variation among homologous proteins occurs at the surface, this procedure will also predict epitopes corresponding to surface-accessible sequences.

Several authors (Tainer et al., 1984; Westhof et al., 1984) have recently suggested that protein epitopes should correspond to regions of high mobility, as defined by X-ray crystallographic temperature factors (Petsko & Ringe, 1984). Mobility is also correlated with surface exposure, since the surface of a protein is generally more flexible than its core and the antigenic determinants recognized by immunoglobulins are indeed surface features (Fanning et al., 1986; Thornton et al., 1986).

The algorithm described by our laboratory (Novotnŷ et al., 1986) and discussed in detail above has been tested successfully on a number of proteins whose structures have been determined by X-ray diffraction and has been found to be more reliable than other proposed methods. To test the ability of the large-probe accessibility algorithm to predict the antigenic epitopes of a molecule whose structure has not yet been determined by X-ray diffraction, we applied the algorithm to a computer model of the three-dimensional structure of renin.

Although the atomic coordinates of human renin have not as yet been determined from X-ray diffraction studies, its three-dimensional structure can be approached by means of a computer model constructed by our laboratory (Carlson et al., 1985), which is based on the crystallographically deter-

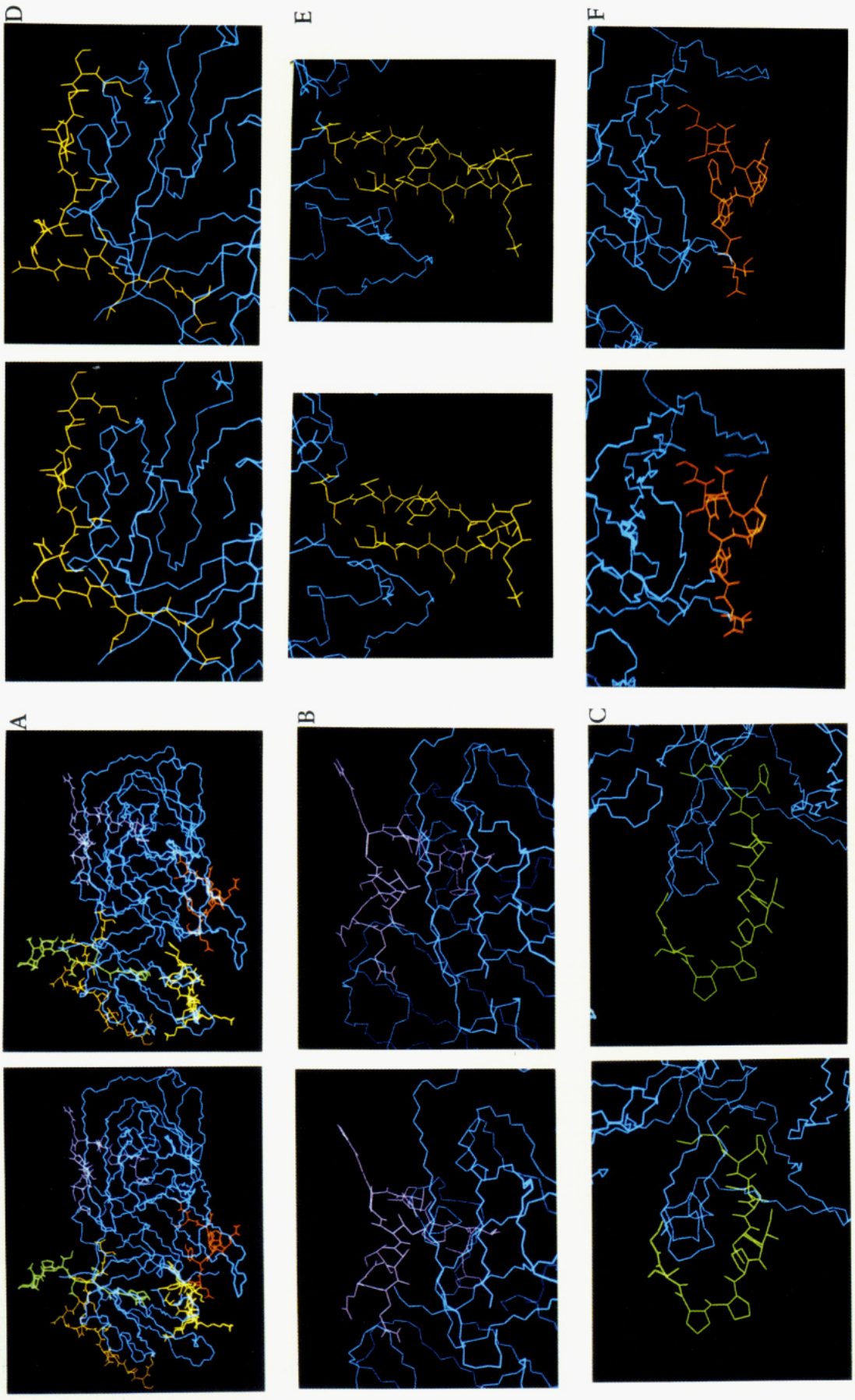

FIGURE 2: Stereoscopic images of the three-dimensional model of renin. (A) Complete structure with peptide backbone shown in blue. Epitopes predicted by the 1-nm probe accessibility algorithm are color coded as follows: (B) epitope 6, Y-133–144 (magenta); (C) epitope 16, Y-300–310 (green); (D) epitope 10, Y-211–224 (orange); (E) epitope 15, 287–297 (yellow); (F) epitope 9, C-180–188 (red). Epitopes are displayed in the conformation they were found to take in the model. The backbone and side chains are shown in a Kendrew-style representation.

mined structures of three other aspartyl proteases. These proteases show a high degree of sequence homology at the core of their three-dimensional structures, whereas there is far less homology at the surface. Similarly, three-dimensional structural homology is highly conserved at the core, near the active site (root mean squared differences in the positions of the peptide backbone atoms of less than 0.5 Å), but is far less conserved at the surface. Indeed, when one compares the sequences of any of these aspartyl proteases, or for that matter the sequences of human renin, mouse renins, cathepsin D, and pepsins, those deletions and insertions that occur do so at positions on the surfaces of the proteins. Because of these structural characteristics, the three-dimensional conformation of the surface residues of our human renin model is likely to be inaccurate in detail. However, given that the three-dimensional structure of human renin is similar in its overall folding to that of the crystallographically studied aspartyl proteases and that the sequence match between renin and these enzymes is correct at the core of the molecules, then the amino acid composition of the surface loops is defined and their precise conformation will have only a secondary effect on their accessibility as calculated here.

Figure 1 shows the plot of the smoothed accessible surface areas of the 17 segments predicted to be epitopes (calculated from the model of the three-dimensional structure of human renin) as a function of the human renin amino acid sequence. Among the 17 peaks, 6 were selected because they correspond to high accessibility values. Figure 2 presents the stereoscopic image of the renin model that shows the location of the accessible regions at the surface of the protein. The regions studied as epitopes are shown in contrasting colors, and the remaining peptide backbone is shown in blue. Peptides that correspond to the selected epitopes were synthesized [Table II; the numbering of the residues refers to the primary structure of mature renin as determined by Imai et al. (1983)]. Peptides whose sequences did not include a tyrosine were synthesized with a tyrosine at the N-terminus to allow ^{125}I labeling.

Peptide Binding to Anti-Renin Antibodies. To assess the validity of epitope prediction, the synthetic peptides were tested for binding to 7 monoclonal and 11 polyclonal antibodies raised against pure human renin. The test was quite significant since these antibodies had been prepared against the native, fully active enzyme. Some peptides were also used as immunogens, and the inhibition of renin enzymatic activity by the resulting antisera was examined. The results of peptide binding studies for 11 polyclonal antibodies using two immunoassay methods are reported in Tables III–V. Peptides corresponding to 5 of the 6 putative epitopes were recognized by at least 1 of the 11 antisera when tested in an enzyme-linked immunosorbent assay (ELISA). The results of the solution assay show recognition of all peptides, except for Y-148–156 and the peptides related to predicted epitope 15 (Table V). Peptides Y-215–224 and Y-211–224 were bound by all 11 anti-renin antisera in the solution assay, indicating that these peptides correspond to a dominant epitope.

It is of interest that the results of the two assay methods did not always correspond. Technical reasons may account for some of these differences. The solution immunoassay requires iodination of the peptide on a tyrosine residue. The required tyrosine was introduced at the amino terminus of all peptides except those relating to epitopes 9 and 15. Peptides of epitope 15, which contains an internal tyrosine, failed to bind in the solution assay yet showed evidence of binding in the ELISA. One possible explanation is that iodination sufficiently altered the structure of the peptide to prevent antibody recognition. In contrast, peptides Y-215–224 and Y-133–144 could be detected by several antibodies in the solution assay but by only 1 of the 11 antibodies in the ELISA. This may be the results of an alteration in the peptide's conformation when it is bound to a plastic surface.

None of the 7 antirenin monoclonal antibodies bound any of the peptides (data not shown). This is not surprising, given the many potential epitopes on renin and the limited number of monoclonal antibodies and peptides tested. There is evidence that members of this set of monoclonal antibodies are directed to at least two different renin epitopes, in that some inhibit renin activity whereas others do not (Galen et al., 1984). However, there is also evidence for overlap among the epitopes that these monoclonal antibodies recognize. For example, 1A12, 2D12, and 3E8 mutually interfere with one another in binding to renin (Galen et al., 1984).

It should also be noted that many protein epitopes are discontinuous, consisting of amino acid segments that are far apart in the primary structure, but which are brought into close

Table IV: Binding of the Synthetic Peptides to 11 Anti-Renin Antibodies Measured by Solution Assay[a]

anti-renin anti-serum	Y-133–144	Y-148–156	C-180–188	Y-215–224	Y-211–224	C-290–296-G	Y-300–310
R11	70[b]	0	0	28	74	0	78
	50[c]			25	65		56
R12	0	0	0	21	63	0	0
				16	45		
R14	0	0	0	33	72	0	0
				32	60		
R15	40	0	0	25	65	0	0
	4			18	52		
R21	0	0	0	27	70	0	15
				23	62		5
R22	70	0	0	24	70	0	88
	63			16	62		78
R24	32	0	53	27	57	0	82
	1		28	17	24		71
R25	0	0	29	27	68	0	75
			8	20	59		66
R26	0	0	51	30	61	0	0
			21	18	31		
Donc	61	0	9	34	65	0	0
	40		2	3	50		
Bof	0	ND[d]	0	26	12	0	0
				19	5		

[a] Results are presented as the percentage of bound tracer. [b] Amount of ^{125}I-labeled peptide bound by a 1:2.5 dilution of antiserum. [c] Amount of ^{125}I-labeled peptide bound by a 1:25 dilution of antiserum. [d] ND, not determined; no more antiserum available.

Table V: Binding Assay of Four Peptides Corresponding to Predicted Epitope 15 Measured by ELISA[a]

anti-renin anti-body	C-290–296-G cyc[b]	C-290–296-G lin[c]	C-287–297 cyc	287–297	285–292	[258–262]-SS-[287–297]
R11	1.8	1.4	1.1	1.1	1.1	1.1
R12	1.5	1.4	1.0	1.0	1.0	1.2
R14	1.4	1.4	1.0	1.7	1.0	1.6
R15	1.3	1.0	1.0	1.2	1.1	1.5
R21	*3.2*	1.3	1.0	1.1	1.1	*2.9*
R22	1.8	1.9	1.0	1.2	1.1	1.3
R24	1.6	1.5	1.0	1.1	1.1	1.3
R25	1.9	1.7	1.1	1.6	1.0	1.3
R26	1.9	1.7	1.0	1.3	1.8	1.1
Donc	*2.8*	1.7	1.1	1.3	1.1	1.6
Bof	*3.0*	ND[d]	1.0	ND	1.4	1.9

[a] Results are presented as the ratio of absorbance (A/A_0) due to antibody (1:500 dilution) binding to peptide and antibody binding in the absence of peptide. [b] cyc = cyclic. [c] lin = linear. [d] ND, not determined; no more antiserum available.

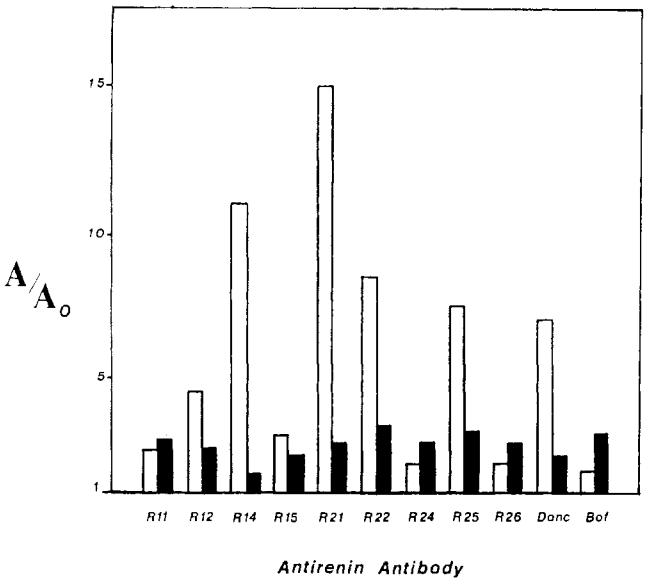

FIGURE 3: Comparative binding of cyclized (open bar) and linear (closed bar) peptide Y-211–224 to 11 anti-renin polyclonal antibodies, as measured by ELISA. A/A_0, ratio of final to initial absorbance at 405 nm.

proximity by folding of the protein backbone (Barlow et al., 1986). Discontinuous epitopes may be visualized as consisting of two or more of the maxima depicted in Figure 1. Monoclonal antibodies prepared against native renin may be directed to such discontinuous epitopes; thus their affinity for sequential fragments that carry only parts of epitopes would be less than their affinity for the entire epitope, and weak binding may not have been detected in our experiments.

Some of the results obtained with the polyclonal anti-renin antibodies bring new insight to our knowledge of the structure of human renin and confirm features of the three-dimensional model. Region 211–224 (shown in orange on Figure 2) was of particular interest. It comprises two cysteines at positions 217 and 221, which, when the model was prepared, were hypothesized to be linked by a disulfide (Carlson et al., 1985). Although there are no homologous cysteine residues in rhizopus pepsin, endothiapepsin, and penicillopepsin, the three aspartyl proteases from which the model was constructed, this disulfide was proposed by homology with porcine pepsin (Sepulveda et al., 1975). It was found that the synthetic peptide Y-211–224, which contains the disulfide loop, was recognized by all 11 antirenin antibodies in the solution assay and by 8 of the 11 in the ELISA. Thus it seems to represent a dominant epitope. A shorter sequence, Y-215–224, was bound by all anti-renin antisera in the solution assay, but less strongly, and was bound by only one antiserum in the ELISA. This suggests that at least one residue of the sequence Gly-Ser-Ser-Thr (211–214) is part of the epitope.

Both peptides Y-211–224 and Y-215–224 were tested after reduction of the disulfide and alkylation of the free cysteines. In the solution assay, the linear peptides Y-215–224 and Y-211–224 were not bound by any of the anti-renin antibodies. In the ELISA, a marked decrease in binding was observed for both peptides (Figure 3 shows the results for Y-211–224). Thus it appears that the epitope is conformationally dependent and that the presence of the disulfide bond between cysteines-217 and -221 is essential for stabilization of the conformation required for epitope recognition. A similar observation has been made by Arnon and Sela (1969), who studied a disulfide-containing epitope of egg-white lysozyme. These authors found that antibodies elicited to lysozyme that were directed to the "loop peptide" were unable to bind the corresponding linear peptide obtained after reduction and carboxymethylation of the cysteines.

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Region 148-156

human renin                    ISQGVLKED
mouse renal renin              L-------E
      subm. renin              L-------K
human cathepsin D              MQ-KLVDQN
human pepsin                   WN--LVSQD


Region 211-224

human renin                    GSSTLLCEDGCLAL
mouse renal renin              --------E--AVV
      subm. renin              --------E--AVV
human cathepsin D              A-GLT--KE--E-I
human pepsin                   NG-AIA-AE--Q-I

Region 132-144

human renin                    FIEQAIGRVTPIF
mouse renal renin              LPA--V-V---V-
      subm. renin              FPA--V-V---V-
human cathepsin D              YPRISVNN-L-V-
human pepsin                   SYYTGSLNWV-VT

Region 300-310

human renin                    IHAMDIPPPTG
mouse renal renin              L----------
      subm. renin              L----------
human cathepsin D              FMG------S-
human pepsin                   FQG-NL-TES-
```

FIGURE 4: Sequence homology among mammalian aspartyl proteases. Subm, submandibular.

Concerning the structure of human renin, our findings strongly support the existence of a disulfide link between cysteines-217 and -221 in renin native structure. The existence of this disulfide was controversial because Misono and Inagami (1982) had reported the presence of free cysteines in mouse submandibular gland renin (without identifying them), and Akahane et al. (1985) have built a model of human renin that depicts cysteines-217 and -221 as free sulfhydryls. Our results appear to refute the latter hypothesis. A corresponding disulfide bond exists in porcine pepsin (Sepulveda et al., 1975) and might also exist in other aspartyl proteases, including cathepsin D. These two cysteines are conserved among mammalian aspartyl proteases, as is a glycine residue preceding the second cysteine, which might accommodate unusual torsion angles to allow formation of the pentapeptidic loop (see Figure 4 for sequence homology with mouse renin and human pepsin and cathepsin D).

Epitope 15 (shown in yellow on Figure 2) was extensively studied. This region finds no direct homology among the aspartyl proteases and thus its precise conformation is quite uncertain. As the surface accessibility value of this particular region of the model was very high, it was rather surprising that the linear peptide 287–297 was not bound by any of the 11 antirenin antisera (Table V). In order to restrain the peptide's conformation and include a bend in the peptide that was apparent in the model, two cyclic peptides were designed by exchanging either Ser-289 (peptide C-290–296-G) or Phe-286 (peptide C-287–297) with a cysteine and creating a disulfide bond with cysteine-296. Of the two cyclic peptides studied, only C-290–296-G was recognized by three anti-renin antibodies and thus appears to possess the conformation necessary for binding to the anti-renin antibodies. We also studied peptide 285–292, which would constitute the C-terminal end of a putative heavy chain in the hypothesis that human renin would be processed in a two-chain structure by cleavage at Lys-293/Lys-294 [by analogy to the processing of mouse submandibular gland renin reported by Panthier et al. (1982)]. Peptide 285–292 was not bound by any anti-renin antibodies. Thus the processing of human renin by cleavage of the peptide backbone in this position appears unlikely.

Table VI: Titers of Antipeptide Antisera As Determined by Solid-Phase and Solution Assays

antiserum	hapten	titer[a] solid-phase assay	titer[a] solution assay
G43	Y-133–144	60	1000
G44	Y-133–144	10	200
G45	C-180–188	400	ND[b]
G46	C-180–188	30	ND
G47	Y-215–224	300	2000
G48	Y-215–224	180	3000
G49	Y-211–224	500	5000
G50	Y-211–224	250	9000
G51	Y-300–310	ND	50000
G52	Y-300–310	ND	110000

[a] Titer values represent antiserum dilutions that bind 50% of either the protein A tracer (solid-phase assay) or the ^{125}I-labeled hapten peptide. [b] ND, not determined.

In an attempt to improve the binding to anti-renin antibodies of peptides of epitope 15, we synthesized a hypothetical discontinuous epitope by linking the open-chain peptide 287–297 through cysteine-296 to peptide 258–262 by means of its cysteine-259 (epitope 13–15, Table II; epitope 13 is not pictured in Figure 2). As suggested in the model and by homology with pepsin, these two cysteines would constitute a cystine, bringing into close proximity residues separated by some 25–30 residues when considered in terms of renin's primary structure. However, this putative discontinuous epitope was bound by only one antibody (compared with three antibodies for cyclic C-290–296-G); therefore, our evidence for the existence of this discontinuous epitope must be considered as only suggestive. Our data demonstrate that epitope 15 at least partially corresponds to an epitope of native renin, but structural information from crystallographic analysis will be required before a better understanding of its precise conformation and extent can be attained.

One of the predicted epitopes, peptide 148–156, failed to bind to any anti-renin antibody. Several factors may account for this negative result. First, the prediction of the epitope may be incorrect because the model is inaccurate. Second, this region may, in the native structure of renin, have a highly restricted conformation that was not incorporated into the synthetic peptide. Third, the rabbits used for immunization with renin may have exhibited an immunologic tolerance to the peptide. In support of the third point, Figure 4 shows the sequence homology among human renin (Imai et al., 1983), mouse renal renin (Holm et al., 1984), mouse submandibular renin (Panthier et al., 1982), human cathepsin D (Faust et al., 1985), and human pepsin (Sogawa et al., 1983). Sequence 148–156 in human renin differs from those in pepsin and cathepsin D, but it is well conserved in submandibular and renal mouse renins (with the exception of the N-terminal and C-terminal residues). The sequence in rabbit renin is not known but may be quite similar to human renin in this particular region. Finally, large probe accessibility may be necessary, but not sufficient, for eliciting an immune response.

Thus, among six regions predicted by the large-probe accessibility algorithm, five were effectively found to correspond to natural epitopes of human renin. However, only a limited number of predicted epitopes have been studied (6 among 17), and no negative controls were included. The question remains whether an equivalent number of peptides chosen to coincide with less exposed regions would have demonstrated comparable binding.

Peptide Immunization. Peptides Y-133–144, C-180–188, Y-215–224, Y-211–224, and Y-300–310 were conjugated to carrier proteins and used to raise polyclonal antibodies in rabbits. Titers were calculated as antibody dilutions binding 50% of tracer. Table VI lists the highest antipeptide titers, obtained after the third booster injection. A large discrepancy appears between titer values obtained from the solid-phase assay and the assay in solution, in that peptide binding seemed to be much more efficient in a solution assay than when the peptide was preadsorbed on a microtiter plate. Peptide Y-300–310 was found to be the most immunogenic; titers of from 1:50 000 to 1:100 000 were obtained for both immunized animals.

Table VII: Inhibition of Human Renin Activity by Purified Antipeptide IgG

IgG	hapten	% inhibition of renin acting on TDP[a]	% inhibition of renin acting on human aogen[b]
G-43	Y-133–144	0	72
G-45	C-180–188	0	0
G-49	Y-211–224	0	33
G-50	Y-211–224	0	0
G-51	Y-300–310	30	28
G-52	Y-300–310	0	0

[a] TDP, synthetic substrate representing the N-terminal tetradecapeptide of human angiotensinogen. [b] aogen, angiotensinogen.

Inhibitory Effect of Antipeptide IgG on Renin Enzymatic Activity. The IgG fractions were purified from antipeptide antisera so as to avoid the interference of other serum components such as renin or angiotensinogen. These fractions were then tested for their ability to inhibit the enzymatic activity of pure human renin when assayed against either pure human angiotensinogen or against a synthetic tetradecapeptide related to its N-terminal sequence (Table VII). IgGs from antisera to peptides Y-133–144, Y-211–224, and Y-300–310 were found to partially inhibit the activity of renin on its natural substrate. The greatest inhibition (72%) was observed with IgG G-43, which is directed to peptide Y-133–144. Only IgG G-51, which was raised against peptide Y-300–310, was able to alter the enzymatic reaction of renin on the synthetic substrate as well as on angiotensinogen (by 30 and 28%, respectively). IgG G-45, anti-C-180–188, did not affect renin activity on either substrate. These results are in agreement with the location of the hapten peptides on the renin model relative to the substrate binding cleft and the enzyme catalytic site. Peptide Y-300–310 is located on the edge of the cleft opposite the flap; antibodies directed to this peptide, like G-51, might thus be expected to block access to the catalytic site. Peptide C-180–188 is located on the side of the molecule opposite the cleft; thus antibodies directed to this peptide would not be expected to inhibit renin activity or to prevent renin substrate from binding. Peptides Y-133–144 and Y-211–224 are not located in the cleft although they are in close proximity; thus, antibodies binding to epitopes that correspond to these peptides would not directly block residues within the cleft involved in renin's catalytic action but might sterically hinder the binding of a macromolecular substrate, angiotensinogen.

We have shown that antigenic determinants of human renin can be predicted from a computer model by calculating surface accessibility to a 1-nm-radius probe. Six regions predicted to be epitopes have been studied with the aid of synthetic peptides and five of them were shown to correspond effectively to antigenic determinants of native renin. The data provide support for the use of homology modeling as a method for approximating surface features even when amino acid sequence homology is low and even though, admittedly, knowledge of the precise conformation awaits a high-resolution crystallo-

graphic study. That conformation plays a role in defining an epitope is demonstrated by a comparison of antibody binding to peptides of restrained and unrestrained structures. Finally, it seems possible to define epitopes near the catalytic site. These would allow for the selection of peptides yielding site-directed antibodies that inhibit enzymatic activity.

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Registry No. Y-133–144, 110613-12-2; Y-148–156, 110567-74-3; C-180–188, 110567-75-4; Y-215–224, 110588-97-1; Y-215–224 (bis-Scm derivative), 110588-98-2; Y-211–224, 110567-76-5; Y-211–224 (bis-Scm derivative), 110567-77-6; C-290–296-G, 104363-25-9; C-290–296-G (bis-Scm derivative), 110567-78-7; C-287–297, 110567-79-8; C-288–297, 110567-80-1; [258–262]-SS-[287–297], 110588-99-3; Y-300–310, 110589-00-9; VFQESYSS, 110567-81-2; renin, 9015-94-5.

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