Interaction of Kidney Renin with Aryl-, Isoalkyl-, and *N*-Alkyl-Substituted Sepharose Derivatives: Multiplicity of Interaction Sites†

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ABSTRACT: Several amino acids occur in high frequency in peptide substrates and inhibitors of kidney renin (EC 3.4.99.19), namely, leucine, valine, phenylalanine, and tyrosine. To examine the functional significance of the individual side chains of these amino acids, the interaction of kidney renin with various small aryl, isoalkyl, and *n*-alkyl ligands was studied by immobilized ligand chromatography and by inhibition of the renin-angiotensinogen reaction. Rabbit kidney renin was selectively and reversibly bound to phenylethylamine- and phenylbutylamine-Sepharoses (50-fold purification), although little activity was bound by aniline- or isoalkylamine-[C_3-C_5]Sepharoses. Within a series of isoalkyl ligands (C_3 to C_9) covalently attached to Sepharose through a hexamethylene spacer arm, renin bound more tightly as the length of the carbon backbone increased. Isobutylamine- and isoamylamine-CH-Sepharoses exhibited higher selectivity for renin over extraneous proteins (150–200-fold purification) than did the *n*-butyl or *n*-hexanoyl derivatives. Free phenylethylamine and the immobilized ligand apparently interact with different sites on renin; both phenylethylamine and phenylethylacetamide in solution appear to compete for the site(s) occupied by immobilized phenylethylamine-Sepharose. However, only phenylethylamine inhibited the renin-angiotensinogen reaction (parabolic competitive kinetics); the acetamide had no effect. The interaction of renin with the isoalkyl-spacer ligand appeared to involve both recognition of the isoalkyl moiety and nonspecific binding to the hexamethylene chain. Free isoamylamine was a noncompetitive (parabolic slope, linear intercept) inhibitor against renin substrate. Thus, renin contains a multiplicity of hydrophobic binding sites which may have significance to in vivo regulation of the enzyme.

Renin (EC 3.4.99.19) is a highly specific proteolytic enzyme which produces angiotensin I from angiotensinogen or its amino-terminal tetradecapeptide by the hydrolysis of a leucyl-leucine bond (Skeggs et al., 1957). There have been several studies of the restrictive substrate specificity of this enzyme and the essential structural requirements necessary for binding to renin. From an analysis of the binding constants and rates of hydrolysis obtained with a series of synthetic peptide substrates resembling fragments of the tetradecapeptide, Skeggs et al. (1968) have shown that the amino-terminal dipeptide, aspartylarginine, is important for binding; however, this dipeptide is eight residues distant from the site of cleavage. The binding requirements at the site of cleavage have been defined by several laboratories from studies of peptide inhibitors containing various amino acid substitutions around the susceptible leucylleucine bond (Kokubu et al., 1973; Poulsen et al., 1973; Parikh & Cuatrecasas, 1973; Workman et al., 1974; Burton et al., 1975). It is apparent from these studies that the most potent inhibitors are those which contain highly lipophilic amino acid side chains in the "cleavage site" region. Further, pepstatin is one of the most potent noncompetitive inhibitors of renin (Aoyagi et al., 1972; Miller et al., 1972; McKown et al., 1974). Although pepstatin does not contain an amino acid sequence which resembles the cleavage site of angiotensinogen, the hydrophobic nature of this pentapeptide points to the importance of such interactions around the binding and/or catalytic site of renin.

We report here a study of the binding properties of kidney renin toward a series of simple hydrophobic ligands, namely, isoalkyl and aryl compounds, which resemble the amino acid side chains found in the cleavage site of the protein substrate and in the more lipophilic inhibitors. We have used immobilized ligand chromatography to determine the extent of selective adsorption of renin from crude kidney homogenates to these simple hydrophobic ligands, and to minimize problems associated with the low solubility of the small hydrophobic molecules in aqueous solution and the denaturing effects of highly nonpolar solvents on protein structure. The kinetics of inhibition of renin by the ligands were analyzed and indicated a multiplicity of binding sites. The results of all these studies indicate that renin can selectively recognize the isoalkyl ligand (isobutyl and isoamyl) as opposed to the *n*-alkyl ligand (*n*-butyl), and that isobutyl, isoamyl, and phenylethyl ligands bound to various Sepharose derivatives may be useful for the purification of renin. Further, inhibition studies as well as competitive elution of renin from covalently immobilized ligands indicate that several types of sites of interaction with free and bound aryl and isoalkyl ligands can be identified on the enzyme surface.

Experimental Procedure

Materials. A crude preparation of rabbit kidney renin was prepared by homogenization of frozen rabbit (White New Zealand) kidneys, low-speed centrifugation to remove cellular debris and ammonium sulfate precipitation, 40% w/v. Following extensive dialysis against 10 mM EDTA, pH 6.0, this preparation of renin was used for all of the chromatographic procedures reported below. Renin used for kinetic studies was further purified by gradient elution from DEAE-cellulose according to Leckie & McConnell (1975) and by affinity chromatography on pepstatin-AH-Sepharose[1] (Inagami & Murakami, 1977). After passage through the pepstatin column, the purified renin was shown to be free of acid protease activity by incubation with 0.5 mL of a 2% denatured hemoglobin solution in 50 mM sodium citrate buffer, pH 3.2, at 37 °C for 90 min (Anson, 1937).

Pepstatin was obtained from Peninsula Laboratories, aniline

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[1] Abbreviations used: CH-Sepharose, carboxyhexamethylene-Sepharose; AH-Sepharose, aminohexamethylene-sepharose; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; AHMHA, 4-amino-3-hydroxy-6-methylheptanoic acid.

0006-2960/78/0417-2484$01.00/0

from Mallinckrodt, phenylethylamine from New England Nuclear, and all other amines used in this study from Aldrich. *N*-Acetyl derivatives of isoamylamine and phenylethylamine were prepared by reaction of the free amine with acetyl chloride, extraction from the reaction mixtures with ethyl ether, and vacuum distillation of the residue after the removal of ether by rotary evaporation: isoamylacetamide, bp 83–86 °C at 0.2 Torr; and phenylethylacetamide, bp 138–139 °C at 0.3 Torr. Phenylbutylamine-Sepharose was purchased from Pierce.

Preparation of Immobilized Ligand Derivatives. Immobilized ligands were prepared with two Sepharose derivatives (Pharmacia). The first series of derivatives was obtained by coupling the following amines to CNBr-activated Sepharose in a solution of 50% dimethylformamide in 100 mM sodium bicarbonate buffer, pH 10.0: aniline and phenylethylamine, (100 mM); isopropylamine, isobutylamine, isoamylamine, and ethanolamine (200 mM). The second group of derivatives was prepared by coupling the following amines to CH-Sepharose; aqueous (100 mM, pH 5–6) solutions of isopropylamine, isobutylamine, isoamylamine, 2-aminoheptane, 1,5-dimethylhexylamine, methylamine, *n*-butylamine, and ethanolamine in the presence of 20 mg/mL 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Bio-Rad); hexadecylamine, octadecylamine, and oleylamine (8 mM, pH 5–6) in dioxane with 10 mg/mL dicyclohexylcarbodiimide (Fisher). Coupling mixtures contained equal volumes of Sepharose and amine solution and were incubated overnight at room temperature on a rotary mixer (4 rpm). The amount of amine derivatives bound to the Sepharose was determined by a trinitrobenzenesulfonic acid assay (McKelvy & Lee, 1969) of the amine concentration free in solution before and after the coupling procedure. Uncoupled amines were washed from the derivatized gel with 30 volumes of aqueous 50% dioxane. Coupling efficiencies for aryl-Sepharose, isoalkyl-Sepharose, and short chain (C_3 to C_9) isoalkyl and *n*-alkyl CH-Sepharose derivatives were between 75 and 95%. Long chain (C_{16} to C_{18}) alkyl CH-Sepharose derivatives contained 2–6 μmol of amine/mL of gel. Comparable coupling efficiencies were obtained for the reaction of [^{14}C]phenylethylamine (New England Nuclear, 50 mCi/mmol) with cyanogen bromide activated Sepharose by measuring the radioisotope bound to the gel after extensive washing. All unbound ^{14}C-labeled reagent was removed from the gel after washing with 20 gel volumes of 50% dioxane.

Chromatography of Renin on Substituted Sepharose Derivatives. Renin activity was eluted from 0.5 × 4.0 cm columns of the various substituted Sepharose derivatives by the stepwise addition of five buffers: (A) equilibration buffer, 20 mM sodium phosphate, pH 6.75; (B) 1 M sodium phosphate, pH 6.75; (C) 100 mM sodium acetate, pH 5.2; (D) 100 mM sodium acetate with 1 M sodium chloride, pH 5.2; and (E) 100 mM acetic acid, pH 3.0. Competitive elution of renin from bound hydrophobic ligands was carried out using 8-mL portions of the same buffers with added free ligands; free ligand was added to the renin solutions prior to application to the immobilized ligand-Sepharose. It was necessary to add 2-methoxyethanol (Aldrich) (1% final concentration) to the elution buffers containing isoamylacetamide, phenylethylamine, and phenylethylacetamide to solubilize these compounds. The effect of this solvent on renin elution was also examined and is reported below. The elution of renin from Sepharose 4B, CH-Sepharose, and AH-Sepharose was also studied to assess the extent of interaction between renin and the dextran backbone or the spacer arm under the chromatographic conditions used here.

Assays of Renin Activity. Enzyme activity was measured by incubation at 37 °C for 60 min of a 25–50-μL sample of the column eluent with 250 μL of a solution containing 0.16 μM hog angiotensinogen (Hog Renin Substrate, Miles-Pentex) in 200 mM sodium phosphate buffer, pH 6.0, with 70 mM phenylmercuric acetate and 30 mM EDTA. The quantity of angiotensin I generated during the incubation was determined by specific radioimmunoassay as previously described (Printz et al., 1977).

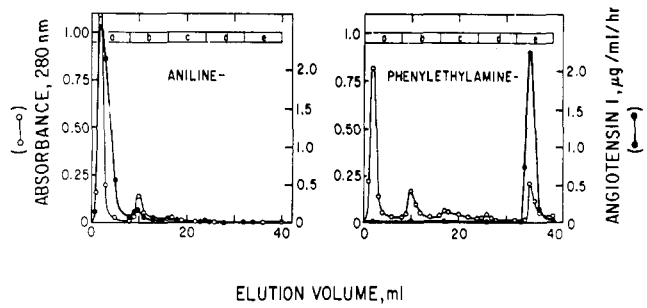

FIGURE 1: Elution of renin activity from aryl-Sepharose derivatives. Crude renin (approximately 8 mg of protein in 10 mM EDTA, pH 6.0) was applied to a 0.5 × 4.0 cm column equilibrated with 20 mM sodium phosphate buffer, pH 6.75, at 22 °C and eluted with the buffers listed in the Experimental Procedure. The letters indicate the buffers used for elution. Flow rates were maintained at 12 mL/h and the fraction volume was 1 mL. The elution profile from phenylbutylamine-Sepharose was similar to that from phenylethylamine-Sepharose.

Inhibition Studies. Studies of the inhibition of the renin–angiotensinogen reaction by the various ligands were carried out under essentially the same conditions as above except that purified renin was used rather than the crude preparation. The more hydrophobic ligands were solubilized with 2-methoxyethanol. The rate of generation of angiotensin I over the 100-min incubation was determined by periodically removing a sample (25 μL) of the incubation mixture and determining the concentration of angiotensin I by radioimmunoassay. The generation of angiotensin I was linear over the 100-min period and the rates were calculated by linear regression analysis ($r > 0.97$). The rates of generation or initial velocities as a function of substrate concentration were fitted to the Michaelis–Menten equation (Cleland, 1967). All *t*-tests for slopes and intercepts were performed according to Johansen & Lumry (1961).

Results

Binding Aryl-Substituted Sepharose Derivatives. The binding of renin to a homologous series of aryl-substituted Sepharoses exhibited a high degree of selectivity with phenylethyl and phenylbutyl ligands (Figure 1); purification of renin was over 50-fold. Renin did not bind to the phenyl ring of aniline–Sepharose.

Binding Isoalkyl- and n-Alkyl-Substituted Sepharose Derivatives. We found that renin could not bind to the isoalkyl ligands (C_3 to C_5) attached directly to the Sepharose backbone, but that the affinity toward these ligands was greatly enhanced if the ligands were attached to Sepharose via a hexamethylene spacer arm (Figure 2). As the length of the carbon chain of the ligand increased (isopropyl to 1,5-dimethylhexyl), a solvent of lower pH and higher ionic strength was required for dissociation of the enzyme from the immobilized ligand. A high degree of selectivity was observed with the isobutyl and isoamyl ligands which resulted in a 150- to 200-fold purification of the major renin peak. Renin, as well as other proteins, bound to 1,5-dimethylhexylamine–CH-Sepharose more tightly than to the shorter chain derivatives. However, in this case, the separation based on the selective recognition of the isoalkyl side-

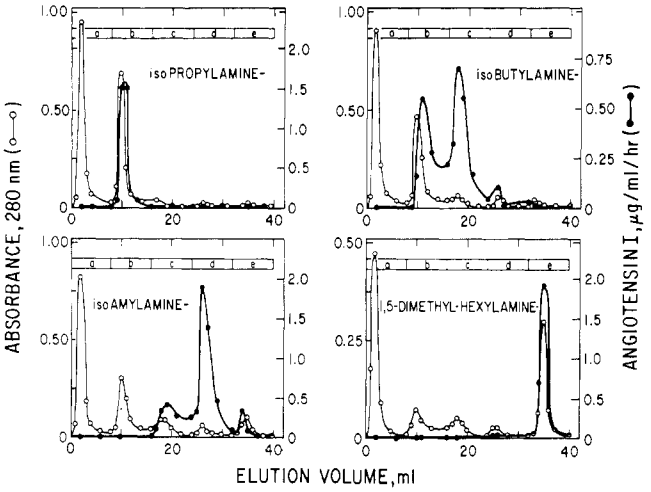

FIGURE 2: Elution of renin activity from isoalkyl-CH-Sepharose derivatives. Crude enzyme (8 mg of protein in 10 mM EDTA, pH 6.0) was chromatographed as outlined in the legend to Figure 1.

arm function by renin was lost and nonspecific hydrophobic interactions with the longer carbon backbone appeared to dominate binding.

Short chain *n*-alkyl-substituted hexamethylene-Sepharose derivatives (butylamine and hexanoic acid) exhibited a lower affinity for renin than the analogous isoalkyl-substituted derivatives (Table I). Enzyme activity was eluted from the butyl and hexanoyl derivatives with milder conditions and in broader peaks which were not distinctly separated as those in Figures 1 and 2. On the other hand, renin bound to long chain alkyl-CH-Sepharoses (hexadecyl, octadecyl, oleyl) was not released by high ionic strength at neutral pH and required the lower pH 3 buffer. The elution patterns of both renin and protein from these latter derivatives were similar to those observed with the 1,5-dimethylhexylamine derivative shown in Figure 2.

Binding Other Sepharose Derivatives. Renin did not interact with the dextran backbone of Sepharose 4B, with ethanolamine-Sepharose or with the anionic carboxyhexamethylene-Sepharose derivative. However, the enzyme did bind weakly to aminohexamethylene-Sepharose, ethanolamine-CH-Sepharose and methylamine-CH-Sepharose since activity was eluted by increasing the ionic strength at neutral pH (buffer B), similar to the pattern observed for isopropylamine-CH-Sepharose (Figure 2). This phenomenon is attributed to nonspecific hydrophobic binding to the alkyl chain of the spacer arm (Shaltiel & Er-el, 1973; Hofstee, 1973).

Recovery of Renin Activity. The total recovery of renin activity after elution from aryl-, isoalkyl- and *n*-alkyl-substituted Sepharose derivatives was complicated by the presence in the crude kidney homogenates of substances which interfered with the renin-angiotensinogen reaction. The columns appeared to remove these substances during chromatography since the magnitudes of the recoveries of renin activity were generally greater than 100%: phenylethylamine-Sepharose, 265%; phenylbutylamine-Sepharose, 276%; ethanolamine-CH-Sepharose, 126%; isopropylamine-CH-Sepharose, 208%; isobutylamine-CH-Sepharose, 314%; and isoamylamine-CH-Sepharose, 393%. The interfering material has not been identified at this time, but could be small hydrophobic inhibitors (molecules or peptides), proteolytic activities which inactivate either renin or angiotensinogen in the assay, or proteases which interfere with the activation of prorenin → renin. In this regard there is evidence that the columns did remove angiotensinases present in the crude homogenate.

TABLE I: Comparison of the Elution Position of Renin from Various *n*-Alkyl and Isoalkyl Substituted Hexamethylene-Sepharose Derivatives.

Ligand	Length of carbon chain in ligand	% total act. released in buffer[a] a	b	c	d	e
n-Alkyl						
Butylamine	4	50	21	29	0	0
Hexanoic acid[b]	6	0	0	22	38	40
2-Aminoheptane	7	0	0	0	16	84
Hexadecylamine	16	1	0	0	1	98
Octadecylamine	18	5	0	0	0	95
Oleylamine	18	5	0	0	0	95
Isoalkyl						
Isobutylamine	3	0	42	53	5	0
Isoamylamine	4	0	0	26	66	8
1,5-Dimethylhexylamine	7	0	0	0	0	100

[a] See Experimental Section for description of buffers. [b] Equal volumes of 100 mM hexanoic acid in 25% dioxane with 40 mg of EDAC/mL and AH-Sepharose were coupled in the same manner as the amines described in the Experimental Section; the amount of coupled hexanoic acid was not determined but it is assumed that it is similar to that of the amine ligands.

Competitive Elution of Renin from Immobilized Ligands. Renin activity was eluted from phenylethylamine-Sepharose and isoamylamine-CH-Sepharose with buffers containing a variety of soluble competing ligands (Tables II and III). The presence of 2-methoxyethanol in the buffers for some experiments had no effect on the elution positions of renin when compared with the aqueous controls. Soluble pepstatin was an effective competitor against the immobilized phenylethyl ligand (Table II) but was only partially effective against the isoamyl ligand (Table III). Free ligands which carried a positive charge displaced renin from isoamylamine-CH-Sepharose, whereas the acetamides of the ligands, which are not charged, were ineffective. These results demonstrate the importance of a positively charged ligand for competition with isoamylamine-CH-Sepharose since those ligands which contain simply bulky hydrophobic groups were only partially effective in displacing renin. In contrast, the hydrophobic nature of the competing ligand was a more important factor for release of renin from phenylethylamine-Sepharose than was ionic charge (Table II). This was clearly evident in the failure of methylamine or isopropylamine to affect release of the enzyme. This aspect of the competitive elution studies is quite different from the results obtained by the inhibition studies (see below) in which the positive charge is an important determinant of ligand-enzyme recognition.

Inhibition of the Renin-Angiotensinogen Reaction. Inhibition of the rates of generation of angiotensin I by purified rabbit renin was studied at varying concentrations (0.025 to 1.25 μM) of hog angiotensinogen and fixed levels of free amines and acetamides. Inhibitor concentrations used for these studies varied between 0 and 200 mM for isoamylamine, isoamylacetamide (in 1% 2-methoxyethanol), and methylamine, and between 0 and 100 mM for phenylethylamine (in 1% 2-methoxyethanol) and phenylethylacetamide (in 2% 2-methoxyethanol). No inhibition was observed with either of the acetamides or with methylamine at any concentration of substrate or reagent tested. Reciprocal plots (Lineweaver-Burk) of the inhibition by phenylethylamine and isoamylamine

TABLE II: Competitive Elution of Renin from Phenylethylamine-Sepharose by Free Ligands.

Free ligand	% act. released at pH 6.75	5.2	3.0
Control, aqueous	0	20	80
Control, 1% 2-methoxyethanol	0	24	76
100 mM methylamine	0	33	67
100 mM isopropylamine	0	26	74
100 mM isoamylamine	0	98	2
100 mM isoamylacetamide[a]	0	93	7
100 mM phenylethylamine[a]	4	96	0
50 mM phenylethylacetamide[a]	1	80	19
100 μM pepstatin[b]	82	17	1
100 mM ethylene glycol	0	28	72

[a] All buffers contain 1% 2-methoxyethanol (126 mM) to solubilize free ligand. [b] After elution of renin from phenylethylamine-Sepharose, the activity was recovered for assay by dialysis against several changes of 100 mM sodium phosphate buffer, pH 7.4, for 36–48 h at 4 °C.

TABLE III: Competitive Elution of Renin from Isoamylamine-CH-Sepharose by Free Ligands.

Free ligand	% act. released at pH 6.75	5.2	3.0
Control, aqueous	2	97	1
Control, 1% 2-methoxyethanol	0	99	1
100 mM methylamine	86	14	0
100 mM isopropylamine	85	14	1
100 mM isoamylamine	80	17	3
100 mM isoamylacetamide[a]	52	48	0
100 mM phenylethylamine[a]	99	1	0
50 mM phenylethylacetamide[a]	10	90	0
100 μM pepstatin[b]	32	68	0
100 mM ethylene glycol	52	48	0

[a] All buffers contain 1% 2-methoxyethanol (126 mM) to solubilize free ligand. [b] After elution of renin from isoamylamine-CH-Sepharose, the activity was recovered for assay by dialysis against several changes of 100 mM sodium phosphate buffer, pH 7.4, for 36–48 h at 4 °C.

are presented in Figures 3 and 4, respectively. Inhibition by phenylethylamine exhibited parabolic competitive kinetics with respect to substrate with a K_m value of 0.40 ± 0.04 μM, whereas inhibition by isoamylamine was nonclassical noncompetitive (the intercept was linear and the slope was parabolic) with a K_m value of 0.57 ± 0.05 μM. Values for the apparent V_{max} differed only slightly due to the presence of 2-methoxyethanol: 1.25 ± 0.07 pg of A_I/h in its presence and 0.84 ± 0.20 pg of A_I/h in its absence. Parabolic inhibition was also observed for both reagents when the data were presented in the form of a Dixon plot, $1/v$ vs. I (Figures 5A and 5B). The initial velocities presented in this manner emphasize deviations from linear inhibition kinetics; the dashed lines in Figures 5A and 5B represent linear extrapolations of the velocities obtained with the lower concentrations of inhibitor. Although these extrapolations are not useful in determining K_I values for parabolic inhibition, they can be used to differentiate the types of inhibition patterns; intersection of the lines above the x axis in Figure 5A is indicative of a competitive inhibition pattern and intersection of the lines on the x axis represents noncompetitive inhibition as in Figure 5B (Dixon, 1953). These inhibition patterns are consistent with a scheme wherein two or more molecules of inhibitor bind to the enzyme.

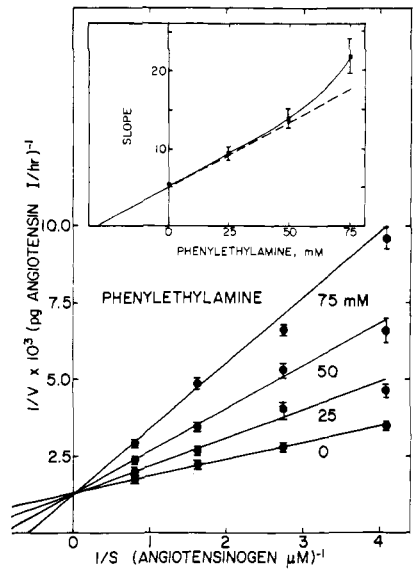

FIGURE 3: Inhibition studies on purified rabbit kidney renin in the presence of varying concentrations of phenylethylamine. Incubations of renin and angiotensinogen were carried out at 37 °C in 1% 2-methoxyethanol in 200 mM sodium phosphate buffer, pH 6.0, containing 30 mM EDTA and 70 mM phenylmercuric acetate. Rates of generation of angiotensin I were determined by assaying aliquots of the incubations by radioimmunoassay at 25, 50, and 100 min. The points and standard deviations shown were derived by linear regression analyses of the angiotensin generation data; the lines shown are least-square fits of the rate data to a hyperbola (Cleland, 1967). A secondary replot of the slopes obtained from the reciprocal plot against the inhibitor concentration is shown as an inset. The dashed line represents a linear extrapolation based on the lower concentrations of inhibitor.

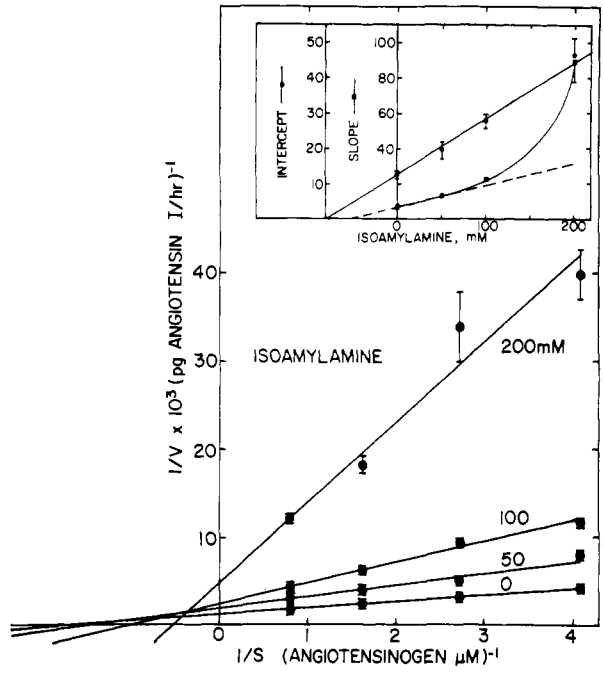

FIGURE 4: Inhibition studies on purified rabbit kidney renin in the presence of varying concentrations of isoamylamine. The studies were performed as described in Figure 3 except that the incubation buffer did not contain 2-methoxyethanol. The inset represents a secondary replot of both the slopes and the intercepts obtained from the reciprocal plot against the inhibitor concentration.

Discussion

The term bioaffinity or biospecific adsorption has been used to describe affinity chromatography which is uncomplicated

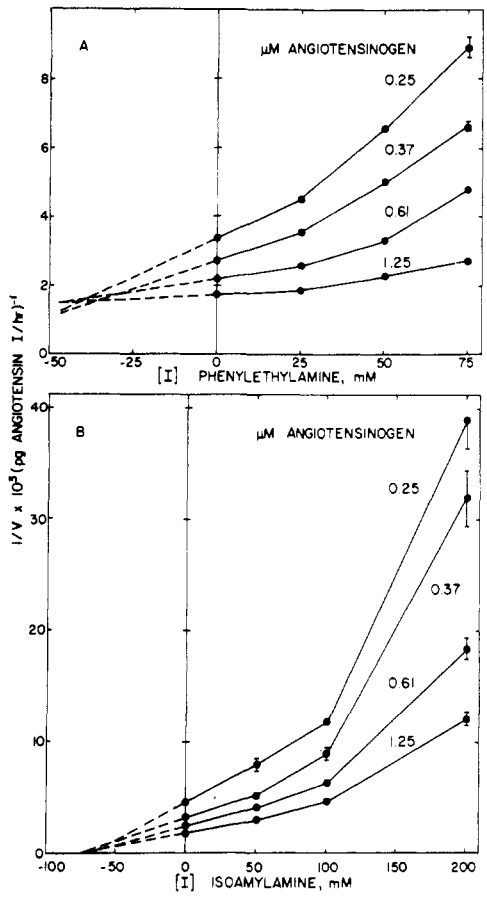

FIGURE 5: Dixon plots of the inhibition of rabbit kidney renin by phenylethylamine and isoamylamine. The descriptions of the inhibition studies are presented in the legends to Figures 3 and 4. The dashed lines represent linear extrapolations of the lines based on the lower concentrations of inhibitor.

by nonspecific adsorption (O'Carra, 1974; O'Carra et al., 1974). A logical approach to the development of a chromatographic model for the purification of renin-like enzymes would be to formulate a binding structure which is similar to peptide inhibitors and substrates of renin. The partial purification of renin using pepstatin (K_i ranging from 10^{-3} to 10^{-10} M depending on the renin species) coupled to AH-Sepharose or to polyacrylamide has been reported by Corvol et al. (1973), Murakami et al. (1973), and Overturf et al. (1974) and more recently has been incorporated into an overall purification scheme with more classical biochemical techniques (Inagami & Murakami, 1977). Synthetic peptide inhibitors resembling the cleavage site (K_i of 3 to 180 μM) have also been used as affinity ligands (Majstoravich et al., 1974; Poulsen et al., 1975). In contrast to these systems the individual side chains of amino acids would not be expected to have a sufficiently unique structure by themselves to provide a high degree of specificity for renin. However, they do have advantages for immobilized chromatography in that they are readily available, inexpensive, and do not require lengthy syntheses. An analysis of those peptides which are good renin substrates and inhibitors indicates that isoalkyl ligands (analogous to the side chains of isoleucine and valine) have a high frequency of occurrence and that both tyrosine and phenylalanine appear to be critical amino acids for both optimum substrate and inhibitor activity.

It was our intent to systematically analyze the relative contribution of selected alkyl, isoalkyl, and aryl ligands which may bind to and/or inhibit renin. It was not clear whether such a simple ligand could recognize and interact with an enzyme which catalyzes the selective hydrolysis of a macromolecular substrate. The specific interaction of two large proteins involves multiple sites of contact, many of which may be necessary for the proper alignment needed for enzymatic activity. Also, a simple ligand could interact reversibly with many sites other than substrate or inhibitor binding sites. The data presented above, however, demonstrate that these simple ligands can interact with renin and exhibit inhibition kinetics which can be readily analyzed.

The inhibition kinetic studies provide insight into the structural characteristics of the ligands which interact with renin. First, inhibition of the renin–angiotensinogen reaction by positively charged phenylethylamine and isoamylamine and a lack of inhibition by comparable concentrations of the acetamide derivatives and methylamine demonstrate that a combination of hydrophobic and ionic features is necessary for binding and inhibition. Second, the individual inhibition patterns for isoamylamine and phenylethylamine indicate that these ligands inhibit by different mechanisms and that two or more molecules of inhibitor interact with sites which participate in binding or catalysis. The aryl group is capable of competing directly with a portion of the protein substrate molecule, and the isoalkyl structure interacts with other site(s) which affect enzyme activity. Despite the noncompetitive inhibition pattern, it is possible that isoamylamine also interacts at site(s) which are competitive against angiotensinogen. Noncompetitive (slope-parabolic) inhibition is consistent with a scheme wherein at least two sites affect the Michaelis constant; only one of these sites can be noncompetitive because the intercept effect is linear. The remaining sites are competitive.

Multiple interactions of phenylethylamine and isoamylamines with the surface of renin can be readily appreciated by recognizing that the tetradecapeptide substrate (Asp-Arg-Tyr-Val-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) binding site may physically accommodate up to six isoalkyl or phenylalkyl side chains. Further, the binding site of pepstatin (isovaleryl-valyl-valyl-AHMHA-alanyl-AHMHA) may accommodate up to five isoalkyl side chains. Thus, parabolic inhibition kinetics which implies multiple binding of inhibitor molecules is not surprising. A second possible source of parabolic inhibition could arise as a result of enzyme denaturation by the high solution concentrations of ligands. This possibility, however, is slight since comparable concentrations of isoamylacetamide, phenylethylacetamide, and methylamine did not inhibit the renin–angiotensinogen reaction and a linear intercept effect was observed with inhibition by isoamylamine. Further, renin bound to the immobilized ligands was not deformed and released by 100 mM solutions of a variety of ligands (see Tables II and III).

Competitive elution studies of renin from isoamylamine–CH-Sepharose indicate that a major contribution to the binding of renin is due to nonspecific adsorption to the hexamethylene spacer arm. Two results which support this hypothesis are the inability of both pepstatin and the acetamides to effectively compete with the immobilized ligand. Since the structure of the acetamides so closely resembles that of the immobilized ligand and its amide linkage, the inability to displace renin must be attributable to the absence of the hexamethylene side chain. Similarly although pepstatin contains two isopropyl and three isobutyl side chains, it is only partially effective. As a result of the recognition of the spacer arm by the enzyme, the contribution of the isoalkyl ligand can be evaluated only in general terms. In particular, selective adsorption of renin by immobilized isobutyl and isoamyl li-

gands and the retention of activity by these hexamethylene derivatives at neutral pH and high ionic strength indicate that the immobilized isoalkyl ligands (C_4 and C_5) do contribute to the binding of renin.

The simplest interpretation of the data obtained from the elution studies with phenylethylamine–Sepharose is that the sites on renin which interact with the bound ligand are different from those which interact with the ligand free in solution. The structure of the bound ligand, an N-substituted isourea, is similar to that of free phenylethylamine in that it still retains a positive charge in close proximity to the amino nitrogen (Axén et al., 1967; Wilchek et al., 1975). However, the equivalent release of renin by both free amines and acetamides indicates that recognition of the positive charge is not necessary for elution. In addition, elution of renin by pepstatin is highly indicative of immobilized ligand interactions at a site which is noncompetitive with angiotensinogen, in contrast to the inhibition by free phenylethylamine. This also is consistent with dissociation of the bound ligand from a site which does not require a positively charged substituent for binding. The predominant feature of those ligands which release renin is a bulky hydrophobic group.

The results of this study indicate that a multiplicity of sites on renin can modify either substrate–enzyme interactions or enzymic activity. The significance of this finding is not diminished by the relatively high concentrations of simple, monofunctional ligands needed since endogenous modifiers may contain multiple functional groups which interact at several sites. Rather, we interpret this data to indicate that this enzyme, which is an important element in the regulation of salt and water balance as well as blood pressure in mammals, is susceptible to multiple mechanisms of regulation in vivo. The implication of these findings to the presence of endogenous modifiers and inhibitors can only be speculated upon at this time.

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