Substrate Specificity of Porcine Renin: P1′, P1, and P3 Residues of Renin Substrates Are Crucial for Activity†

Wei Wang and T. Chyau Liang*

Department of Biochemistry and Molecular Biology, University of Texas Medical School, P.O. Box 20708, Houston, Texas 77225

Received April 21, 1994; Revised Manuscript Received September 1, 1994®

ABSTRACT: Renin, the rate-limiting enzyme in the formation of angiotensin II, is well-known for its stringent substrate specificity. In this study, the biochemical basis for the unusual specificity of renin was investigated by replacing individual amino acids in the octapeptide substrate of renin with Ala. Kinetic analyses of Ala-substituted substrates revealed that the substitutions did not cause significant changes in the K_m values, but did cause variable changes in the k_{cat} and k_{cat}/K_m values. Ala substitutions at the P1′, P1, and P3 sites decreased the k_{cat}/K_m values by 400−700-fold. Similar substitutions at the P3′, P2, P4, and P5 sites only reduced the k_{cat}/K_m values by 2−7-fold. Interestingly, Ala substitution for the P2′ Val produced a substrate with an approximately 3-fold increase in activity. These results indicate that the P1′, P1, and P3 residues are crucial in determining the substrate specificity of renin. The findings also suggest that the specificity of renin is achieved mainly through substrate discrimination in the transition state, rather than in the ground state. Further studies on the effects of amino acid substitutions at the P2′ site revealed that non-branched-chain amino acids (e.g., Ala and α-aminobutyric acid) are preferred at this site. Only P1′ substitution demonstrated any significant change in K_m, presumably due to the decreased hydrophobic interactions in the S1′ site upon Ala substitution. The species specificity of renin presuambly arises from differing P1′−P3′ residues in angiotensinogens. For example, the P1′−P3′ residues from human and porcine angiotensinogens are Ile-Val-His and Leu-Val-Tyr, respectively. Replacing the P1′ Leu with Val in a substrate of porcine renin resulted in a 78-fold decrease in activity, while replacing the P2′ Val with Ile had little effect. This result suggests that the P1′ residue is more important than the P2′ residue in determining the species specificity of renin.

Renin (EC 3.4.99.19) is the rate-limiting enzyme in the production of angiotensin II. By hydrolyzing angiotensinogen between residues 10 and 11, the N-terminal decapeptide, angiotensin I, is released. Angiotensin I, in turn, is cleaved by the angiotensin-converting enzyme to generate angiotensin II, a potent vasoconstrictor. Angiotensin II initiates vasoconstriction by binding to receptors in blood vessels. In addition, it also affects renal sodium and water retention, leading to higher blood pressure. Due to the pharmacological importance of renin, there has been intense interest in the design of renin inhibitors [for a review see Greenlee (1990)]. In comparison, relatively little effort has been devoted to the biochemical characterization of this important enzyme. Renin is known for its unusual specificity [for reviews see: Murakami et al. (1979), Inagami et al. (1978 and 1980), Inagami (1981 and 1989), and Hutchins and Greer (1991)]. Angiotensinogen is the only natural substrate for renin. Furthermore, renin has a pH optimum around neutrality, whereas other aspartic proteases have acidic pH optima due to the involvement of two essential aspartic acids functioning as a general acid and a general base. The biochemical basis for these unusual properties of renin is not clear.

Skeggs et al. (1968) demonstrated that the minimal substrate of renin is an octapeptide corresponding to the cleavage site sequence in angiotensinogen. Consistent with this result, X-ray (Sielecki et al., 1989; Dhanaraj et al., 1992) and modeling studies (Blundell et al., 1983; Sibanda et al., 1984 and 1985; Akahane et al., 1985; Carlson et al., 1985; Hemmings et al., 1985) reveal that renin, like other aspartic proteases, has an extensive active site consisting of eight or more binding subsites. The large number of subsites alone does not explain why renin is highly specific. For example, pepsin also has a large number of binding subsites, yet it is among the least specific proteases. *A priori*, a specific protease recognizes its substrate by the unique array of amino acid side chains rather than by the ubiquitous backbone amides. Therefore, the exquisite specificity of renin must arise from specific interactions between the side chains of the octapeptide and their corresponding binding subsites. It is not known, however, whether all eight amino acids in the octapeptide are important for specific interactions with renin. Studies on other specific proteases, such as factor Xa, thrombin, and enterokinase, indicate that these proteases recognize their substrates by unique sequences of 3−5 amino acids. Therefore, it is unlikely that all eight amino acids in the octapeptide are important for specific interactions with renin, and the question remains as to which amino acids are important.

Renin is also known to be species specific. For example, human angiotensinogen is a poor substrate for porcine renin. The species specificity presumably arises from differing sequences at the P1′−P3′ sites, since the residues at the P5−P1 sites are identical among all angiotensinogens [Tewksbury

† Supported by a Grant-In-Aid from the American Heart Association, Texas Affiliate, Inc.

* Address correspondence to this author. Telephone: (713) 792-5265. FAX: (713) 794-4150.

® Abstract published in *Advance ACS Abstracts,* November 1, 1994.

0006-2960/94/0433-14636$04.50/0

Table 1: Kinetic Constants of Alanine-Substituted Renin Substrates[a]

peptide	sequence	K_m or K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})
F	HPWHLLAF(NO_2)	19.5 ± 2.54	0.56 ± 0.04	28.7 ± 5.5
0	Ac-HPFHLLVYS	46.9 ± 13.46	0.59 ± 0.06	12.5 ± 4.9
P3′	Ac-HPFHLLVAS	51.2 ± 6.81[b]	0.09 ± 0.01	1.8 ± 0.3
P2′	Ac-HPFHLLAYS	41.5 ± 3.91	1.98 ± 0.65	47.7 ± 20.2
P1′	Ac-HPFHLAVYS	284.1 ± 80.39[b]	0.0065 ± 0.0002	0.023 ± 0.007
P1	Ac-HPFHALVYS	49.6 ± 6.33[b]	0.0016 ± 0.0001	0.032 ± 0.006
P2	Ac-HPFALLVYS	46.0 ± 0.75[b]	0.24 ± 0.008	5.3 ± 0.3
P3	Ac-HPAHLLVYS	33.1 ± 1.28[b]	0.0006 ± 0.0001	0.018 ± 0.002
P4	Ac-HAFHLLVYS	51.2 ± 7.41[b]	0.51 ± 0.02	9.9 ± 1.8
P5	Ac-APFHLLVYS	88.7 ± 15.24[b]	0.70 ± 0.01	7.8 ± 1.4

[a] Assays were performed in 50 mM MOPS (pH 6.8) at 37 °C. See Materials and Methods for details. [b] K_i values were determined with a fluorogenic substrate.

et al., 1979; for P*n* and P*n*′ nomenclature, see Schechter and Berger (1968)]. However, it is not clear which of the residues in the P1′−P3′ sites is more important for the species specificity of renin.

To answer these questions and to elucidate the biochemical basis for the unusual specificity of renin, we have investigated the importance of each amino acid in the octapeptide by replacing each of them with Ala. Kinetic studies using these Ala-substituted peptides revealed that only three amino acids are crucial for activity. Ala substitution at the P2′ site produced a better substrate, indicating that the original octapeptide is not the optimal sequence to interact with renin. Furthermore, by analyzing the effects of amino acid substitutions at the P1′ and P2′ sites, we have shown that the P1′ residue is more important than the P2′ residue in determining the species specificity of renin.

MATERIALS AND METHODS

Merrifield peptide resin and BOC[1] -protected amino acids were purchased from Advanced ChemTech (Louisville, KY). Trifluoroacetic acid was purchased from Halocarbon Laboratories, Inc. (River Edge, NJ). Dicyclohexylcarbodiimide, L-*p*-nitrophenylalanine, di-*tert*-butyl dicarbonate, *N*-hydroxysuccinimide, acetic anhydride, triethylamine, 1-hydroxybenzotriazole, thioanisole, ethylenedithiol, trifluoromethanesulfonic acid, thiophenol, and fluorescamine were obtained from Aldrich Chemical Co. (Milwaukee, WI). Porcine kidney renin[2] and MOPS were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin was purchased from Miles Laboratories (Naperville, IL). All solvents and other reagents were from commercial sources and were used without further purification, except for dimethylformamide, which was dried with molecular sieves before use.

Peptide Synthesis. Peptides used in this study were prepared by the standard solid-phase method using Merrifield resin and BOC amino acids (Barany & Merrifield, 1978; Steward & Young, 1984). The following side-chain-protected amino acids were used: BOC-Ser(Bzl), BOC-Tyr(Cl_2-Bzl), and BOC-His(DNP). All coupling steps were carried out with 2.5 equiv each of BOC-amino acids, DCC, and HOBT. The DNP protecting group on His was removed with thiophenol before the peptides were cleaved from the resin. Peptides were cleaved from the resins with trifluoromethanesulfonic acid in the presence of thioanisole, ethylenedithiol, and TFA. The crude peptides were first precipitated with ethyl ether and then purified on a reverse-phase LoBar C-18 column (E. Mercks; 3.0 × 31 cm). The purities of these peptides were analyzed by HPLC. The identities of these peptides were confirmed by ^{1}H NMR spectroscopy and/or FAB-MS.

Renin Activity Assays. Hydrolyses of the synthetic peptide substrates by renin were assayed with fluorescamine according to the procedures of Galen et al. (1978). Briefly, the N-acetylated peptides (10−200 μM) were incubated with porcine renin (0.2−100 milliunits) at 37 °C in 50 mM MOPS buffer (pH 6.8) containing 0.18 mg/mL acetylated bovine serum albumin to prevent the loss of enzymatic activity over time. This concentration of the acetylated BSA was found to protect renin activity for over 20 h. Without the added BSA, renin activity decreased appreciably within 2 h. At various intervals, aliquots (150 μL) were withdrawn and heated in boiling water for 5 min to stop the enzymatic reactions. To the inactivated, cooled reaction mixture was added 700 μL of saturated sodium borate solution (pH 9.3), followed by 150 μL of fluorescamine in acetone (0.3 mg/mL). The fluorescence intensities of these solutions were then determined with an Aminco-Bowman spectrofluorometer (excitation, 390 nm; emission, 490 nm). These relative fluorescence readings as a function of time were then used to construct hydrolysis progress curves. The initial linear portions of these curves were used to calculate the initial rates of the enzymatic reactions. For peptide 0 and peptide P2′ (Table 1), these initial rates were then fit to the Michaelis−Menton equation to give k_{cat} and K_m values using either the MINSQ program (version 4.0, MicroMath, Salt Lake City, UT) or the Enzyme Kinetics program (version 1.0c) written by Dr. D. G. Gilbert of Indiana University. The hydrolysis rates for other peptides, especially peptides P1′, P1, and P3, were slow. It was more convenient to determine their K_i values by assaying them as inhibitors of a fluorogenic

[1] Abbreviations: BOC, *tert*-butyloxycarbonyl; Bzl, benzyl; Cl_2-Bzl, 2,6-dichlorobenzyl; DCC, dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole; BSA, bovine serum albumin; DNP, dinitrophenyl; TFA, trifluoroacetic acid; MOPS, 3-morpholinopropanesulfonic acid; NMR, nuclear magnetic resonance; FAB-MS, fast atom bombardment mass spectrometry. Standard three-letter amino acid codes are used except for two special amino acids: Phe(NO_2), *p*-nitrophenylalanine; Abu, α-aminobutyric acid.

[2] One unit of porcine renin releases 100 μg of angiotensin I per hour from angiotensinogen at pH 6.0 and 37 °C. According to Inagami and Murakami (1977),1 mg of porcine renin is equivalent to 2000 Goldblatt units, and the molecular weight of porcine renin is 36 400. Thus, 1 unit of renin activity is equivalent to 1.37×10^{-11} mol of enzyme. The k_{cat} values reported in this study are based on this conversion factor. The renin preparation was confirmed to have no contaminating protease activities on the basis of the following criteria: (i) the observed activity was specifically inhibited by a difluorostatone-containing renin inhibitor (Fearon et al., 1987) with a K_i of 7 nM (T. C. Liang, unpublished result); (ii) the observed activity was not affected by the addition of PMSF or EDTA.

substrate (see the following) than to obtain their K_m values with multiple substrate concentrations. It has been shown with many proteases acting on amide substrates that the K_m values should be approximately equal to their K_s values, which in turn are approximately equal to the K_i values when these peptides are assayed as inhibitors of the enzyme. With peptides 0 and P2′, we have verified that their K_m and K_i values were identical within experimental errors. Therefore, the K_i values determined for other peptides could be treated as approximate values for their corresponding K_m values. The k_{cat} values of these peptides could then be calculated from the initial rates of hydrolysis and the approximate K_m values using the Michaelis−Menten equation.

Determination of Inhibition Constants Using a Fluorescence Assay. Inhibitions of renin by Ala-substituted peptides were assayed using a fluorogenic peptide, His-Pro-Trp-His-Leu-Leu-Ala-Phe(NO_2), in the same MOPS buffer (Wang & Liang, 1994). This fluorogenic substrate of renin was prepared by replacing tryptophan and *p*-nitrophenylalanine for Phe and Tyr, respectively, in the minimal octapeptide. The fluorescence of tryptophan (excitation, 290 nm; emission, 355 nm) is quenched by *p*-nitrophenylalanine in this peptide. Hydrolysis of this peptide by renin between the two Leu residues relieves this quenching and results in an increase in tryptophan fluorescence. This substrate provided a convenient assay for renin activity. Various concentrations (20−200 μM) of the Ala-substituted peptides were added to the assay solution containing various concentrations (5−40 μM) of the fluorogenic substrate. The initial rates of hydrolysis were calculated from the linear portions of the hydrolysis progress curves and used in double-reciprocal plots to arrive at the inhibition constants (K_i) of these peptides.

Assay of Relative Reactivities of P2′-Substituted Peptides. Peptides with various amino acid substitutions at the P2′ sites were assayed for their relative reactivities by using 10 μM each of these peptides and 0.2 milliunit of porcine renin in a total volume of 1 mL of MOPS buffer (50 mM, pH 6.8) containing 0.18 mg/mL acetylated BSA. At various intervals, aliquots were withdrawn and reacted with fluorescamine as described earlier. The fluorescence intensities of these aliquots were determined. For each peptide, the fluorescence increases as a function of time were plotted to give the hydrolysis progress curves. The initial linear portions of these curves were used to calculate the initial rates of enzymatic reactions. These rates were then compared with each other to give the relative activities of these peptides. Since most Ala-substituted peptides have either unchanged or higher K_m values, it is reasonable to assume that these various P2′-substituted peptides will have $K_m \geq 40$ μM. Therefore, these peptides were assayed under the conditions of $[S] < K_m$. As a result, the Michaelis−Menten equation, $v = V_{max}[S]/(K_m + [S])$, can be simplified to $v \cong V_{max}[S]/K_m$. Therefore, the relative rates (v) obtained from this experiment can be used as a direct comparison for their relative V_{max}/K_m or k_{cat}/K_m values.

RESULTS

Effects of Alanine Substitution in the Renin Substrate. Skeggs et al. (1968) showed that the minimal substrate of renin is an octapeptide derived from residues 6−13 of angiotensinogen. According to the nomenclature of Schechter and Berger (1968), this minimal peptide corresponds to the P5−P3′ residues of a renin substrate. For porcine renin, this minimal substrate sequence is His-Pro-Phe-His-Leu-Leu-Val-Tyr. In this study, we included an extra Ser, corresponding to the P4′ residue, at the C-termini of all peptides to increase their solubilities and to facilitate synthesis of these peptides from the same BOC-Ser(Bzl) resin. Inclusion of this residue should not affect the kinetic constants (Skeggs et al., 1968).

To evaluate the contribution of each amino acid in the octapeptide substrate of renin, we have systematically replaced each of them with Ala. In so doing, we essentially removed parts of the side chain beyond the β-carbon. Any changes in the activities of the resultant peptides can then be ascribed to contributions from the deleted portions of the side chains. Table 1 summarizes the kinetic constants of these Ala-substituted substrates of renin.

Except for peptide P1′ ($K_i = 284.1$ μM), Ala substitutions did not significantly alter the K_m or K_i values of the resultant peptides (33.1−88.7 μM vs 46.9 μM for the original peptide). The mechanism for the higher K_m upon P1′ substitution was investigated further by substitutions with other amino acids (see the following). The fact that the K_m or K_i values were not significantly affected by Ala substitutions at sites other than P1′ suggests that these individual amino acid side chains did not contribute significantly to the ground-state interactions in the formation of Michaelis complexes.

In contrast to the minimal effects on the K_m values, Ala substitutions in the renin substrate produced variable effects on the k_{cat} and k_{cat}/K_m values of these peptides. For peptides P2, P4, and P5, their k_{cat} values (0.24, 0.51, and 0.70 s^{-1}, respectively) are not significantly different from that of the original peptide (0.59 s^{-1}). Ala substitution at P3′ resulted in a moderate reduction in the k_{cat} value (0.09 s^{-1}). In contrast, Ala substitutions at P1′, P1, and P3 all resulted in dramatically reduced k_{cat} values (0.0065, 0.0016, and 0.0006 s^{-1}, respectively), suggesting that these three residues are crucial for interactions in the transition state.

The changes in k_{cat}/K_m values in general paralleled the changes in k_{cat} values, since the K_m values change little in most peptides. Thus, the k_{cat}/K_m values of peptides P2 (5.3×10^3 M^{-1} s^{-1}), P4 (9.9×10^3 M^{-1} s^{-1}), and P5 (7.8×10^3 M^{-1} s^{-1}) are not significantly different from that of the original substrate (1.25×10^4 M^{-1} s^{-1}). Ala substitution at P3′ reduced the k_{cat}/K_m (1.8×10^3 M^{-1} s^{-1}) by less than 7-fold. In contrast, Ala substitutions at P1′ ($k_{cat}/K_m = 23$ M^{-1} s^{-1}), P1 ($k_{cat}/K_m = 32$ M^{-1} s^{-1}), and P3 ($k_{cat}/K_m = 18$ M^{-1} s^{-1}) reduced the activities of these peptides by a factor of 400−700.

Surprisingly, Ala substitution at P2′ resulted in a substrate with k_{cat} (1.98 s^{-1}) and k_{cat}/K_m (4.77×10^4 M^{-1} s^{-1}) values almost 4 times those of the original peptide. The mechanism for this improvement was investigated further by replacing the P2′ residue with other amino acids (see the following).

Effects of Amino Acid Substitutions at the P1′ Site. The fact that Ala substitution at the P1′ site changed the K_m value suggests a special role for the P1′ Leu in the ground-state interactions. To investigate this possibility, we carried out additional amino acid substitutions at this site. Results from this study are summarized in Table 2. In general, amino acids with smaller side chains (Gly, Ala, Val) give higher K_m values (141.8−284.1 μM), while amino acids with large side chains (Phe and Ile: 36.1 and 38.3 μM, respectively) do not significantly change the K_m values of the resultant peptides. A similar size-dependent effect was also observed for the k_{cat} values. For example, the k_{cat} values for peptides

Table 2: Kinetic Constants of P1′-Substituted Renin Substrates[a]

peptide	sequence	K_m or K_i (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})
0	Ac-HPFHLLVYS	46.9 ± 13.4	0.59 ± 0.06	12.5 ± 4.9
P1′	Ac-HPFHLAVYS	284.1 ± 80.4[b]	0.0065 ± 0.0002	0.023 ± 0.007
P1′ Gly	Ac-HPFHLGVYS	276.0 ± 39.4[b]	0.016 ± 0.001	0.059 ± 0.01
P1′ Val	Ac-HPFHLVVYS	141.8 ± 36.6[b]	0.023 ± 0.001	0.16 ± 0.04
P1′ Ile	Ac-HPFHLIVYS	38.3 ± 10.9[b]	0.13 ± 0.01	3.5 ± 1.3
P1′ Phe	Ac-HPFHLFVYS	36.1 ± 2.0[b]	0.12 ± 0.01	3.4 ± 0.4
P1 Phe	Ac-HPFHFLVYS	11.4 ± 0.5[b]	0.34 ± 0.05	30.0 ± 1.8
P1,1′ Phe	Ac-HPFHFFVYS	13.2 ± 0.3[b]	0.086 ± 0.011	6.5 ± 0.9

[a] Assays were performed in 50 mM MOPS (pH 6.8) at 37 °C. See Materials and Methods for details. [b] K_i values were determined with a fluorogenic substrate.

containing Ala, Gly, and Val are 0.0065, 0.016, and 0.023 s^{-1}, respectively, while those for peptides containing Phe and Ile are 0.12 and 0.13 s^{-1}, respectively. These results suggest that the S1′ subsite is relatively large and hydrophobic. The combination of changes in K_m and k_{cat} resulted in even more dramatic changes in the k_{cat}/K_m values (Table 2). For example, Val substitution at this site decreased the activity of the resultant peptide (k_{cat}/K_m = 1.6 × 10^2 M^{-1} s^{-1}) by almost 2 orders of magnitude. Incidentally, human angiotensinogen, which contains a Val instead of a Leu at this site, is a poor substrate for porcine renin. This result suggests that the P1′ residue may be a major determinant of the species specificity of renin.

Phe substitution for the P1′ Leu (Table 2, peptide P1′ Phe) resulted in little change in K_m (36.1 vs 46.9 μM for peptide 0), but a significant reduction in k_{cat} (0.12 vs 0.59 s^{-1} for peptide 0). These changes gave peptide P1′ Phe a k_{cat}/K_m value of about one-third that of the original peptide. This result is interesting in light of reports that peptides containing Phe at both the P1 and P1′ sites were inhibitors of human renin (Poulsen et al., 1976; Burton et al., 1980; Cody et al., 1980). Although the inhibition study was not performed with porcine renin, it is likely that similar Phe substitutions in a porcine substrate may produce a similar effect. The fact that a single Phe substitution at the P1′ site only slightly reduced its overall k_{cat}/K_m value prompted us to examine the effects of Phe substitution at the P1 site as well as Phe substitutions at both the P1 and P1′ sites. As shown in Table 2, a single Phe substitution for the P1 Leu (peptide P1 Phe) resulted in a 75% reduction in K_m (11.4 vs 46.9 μM) and a 40% reduction in k_{cat} (0.34 vs 0.59 s^{-1}). As a result, this peptide has a slightly higher k_{cat}/K_m value than the original peptide (3.0 × 10^4 vs 1.25 × 10^4 M^{-1} s^{-1}). It is interesting that Phe substitution at the P1 site produced a greater effect on K_m, while Phe substitution at the P1′ site had a greater effect on k_{cat}. When both of the Leu residues at the P1 and P1′ sites were replaced by Phe, the resultant peptide (peptide P1,1′ Phe) had a lower K_m (13.2 vs 46.9 μM) and a lower k_{cat} (0.086 vs 0.59 s^{-1}). These changes seem to result from the additive effects of Phe substitutions at these sites. Due to the lower K_m, this double-Phe-substituted peptide has a better affinity for renin. On the other hand, due to the lower k_{cat}, this peptide will be hydrolyzed at only one-seventh the rate once it is bound in the active site. These two effects together explain why the double-Phe-substituted peptide is an inhibitor of renin.

Effects of Other Amino Acid Substitutions at the P2′ Site. The observation that Ala substitution for the P2′ Val increased the activity of the peptide is intriguing. The mechanism for this enhancement was investigated with peptides containing other amino acids at this site. As shown in Table 3, substitution of the P2′ Val by Leu or Ile resulted in no detectable changes in the activities of these peptides. The fact that replacing P2′ Val with Ile had little effect is in contrast to the marked reduction in activity when P1′ Leu is replaced with Val, suggesting that the presence of Val at the P1′ site, but not Ile at the P2′ site, in human angiotensinogen is responsible for the inability of porcine renin to hydrolyze human angiotensinogen efficiently.

Table 3: Relative Activities of P2′-Substituted Renin Substrates[a]

peptide	sequence	relative rate[b]
0	Ac-HPFHLLVYS	1.0
P2′	Ac-HPFHLLAYS	2.9 ± 0.4
P2′ Gly	Ac-HPFHLLGYS	0.7 ± 0.1
P2′ Abu[c]	Ac-HPFHLLBYS	2.9 ± 0.4
P2′ Ile	Ac-HPFHLLIYS	0.98 ± 0.07
P2′ Leu	Ac-HPFHLLLYS	0.96 ± 0.09
P2′ Tyr	Ac-HPFHLLYYS	1.3 ± 0.1

[a] Assays were performed in 50 mM MOPS (pH 6.8) at 37 °C. See Materials and Methods for details. [b] Relative rates were assayed under the conditions of [S] < K_m. Therefore, the relative rates should reflect the relative k_{cat}/K_m values. [c] α-Aminobutyric acid; indicated as B in the sequence.

Replacment of P2′ Val with Gly produced a 30% drop in activity, while its replacement with Tyr increased the activity by 30%. Significant enhancement (about 3-fold) of activities was observed when P2′ Val was replaced with Ala and α-aminobutyric acid, suggesting that non-branched-chain amino acids are preferred at this site.

DISCUSSION

Skeggs et al. (1968) first characterized the specificity of renin using peptides prepared according to the cleavage site sequence of angiotensinogen. By shortening the peptides from both ends, they found that the minimal substrate for renin was an octapeptide corresponding to residues 6−13 of angiotensinogen. However, this result does not mean that all eight amino acids in the octapeptide are important for activity, because by removing amino acids from either end of the octapeptide, one brings the charge closer to the cleavage site. As a result, the decreased activities of shorter peptides may result from unfavorable charge interactions rather than from the loss of N- and C-terminal residues. With the Ala scanning method, one can avoid this complication. Results from our study show that Ala substitutions for the P1′ Leu, P1 Leu, and P3 Phe all resulted in a reduction in the activity by a factor of more than 2 orders of magnitude, suggesting that these three residues are crucial for activity. In comparison, Ala substitutions for the P3′ Tyr, P2 His, P4 Pro, and P5 His only resulted in slightly decreased activities, while Ala substitution for P2′ Val unexpectedly improved the activity of the resultant peptide.

The finding that only P3, P1, and P1′ residues are crucial for activity is fairly consistent with the structural information available from recent X-ray crystallographic studies on human and mouse renins complexed with substrate analogs (Dhanaraj et al., 1992; Dealwis et al., 1994). These studies indicate that the S5 subsite is located on the surface of the N-terminal lobe, and the P5 His bound at this site is fairly accessible to solvent. Consistent with this finding, our result indicates that the P5 residue is not important for renin−substrate interaction. The X-ray structure of the renin−inhibitor complex shows that the P4 Pro lies between Phe220 and His287 and the main-chain amide interacts with Ser219. Ala substitution at this site will retain the interaction with Ser219, while the methyl side chain can still fit in the S4 pocket. Therefore, Ala substitution at the P4 site should produce a minimal effect.

The S3−S1′ subsites are found to be compact and inaccessible to solvent. Particularly, the P3 and P1′ residues make many van der Waals contacts in their respective binding sites. Our finding that the P3, P1, and P1′ residues are crucial is consistent with this structural information. On the other hand, the unimportance of P2 His, as suggested by our kinetic study and several other inibition studies (Rosenberg et al., 1987; Thaisrivongs et al., 1990; Doherty et al., 1991), may seem to contradict the finding that P2 His fits nicely in the S2 site and a hydrogen bond forms between imidazole and Ser222. The seemingly contradictory conclusions inferred from kinetic and X-ray studies may be reconciled by the fact that a stretch of peptide (residues 277−284) with high temperature factors precedes the stretch (residues 287−291) lining the S2 site. The presence of this stretch with high temperature factors suggests that the S2 site may be flexible and can adapt to different P2 residues.

The crystallographic data from human and mouse renins indicate that the S2′ subsite is quite shallow and that the P2′ residue bound is accessible to solvent. In addition, the P3′ residue in mouse renin is also fairly accessible to solvent. This is consistent with our finding that Ala substitution for the P2′ Val or the P3′ Tyr did not significantly compromise the activity of the peptide.

The kinetic constants (K_m, k_{cat}, and k_{cat}/K_m) of an enzymatic reaction can often provide valuable insights into the mechanism of enzyme−substrate interactions. With regard to protease-catalyzed hydrolysis of peptide substrates, one often finds similar K_s and K_m values for a specific substrate. This is because formation of the initial tetrahedral transition state is often the rate-limiting step. Fruton (1970) showed that for aspartic proteases acting on peptide substrates, the K_m values are identical to their corresponding K_s values, which in turn are approximately the same as the K_i values when these peptides are assayed as inhibitors. We have also verified that the K_m values of peptides 0 and P2′ are indistinguishable from their respective K_i values when these peptides are assayed as competitive inhibitors of a fluorogenic substrate, suggesting that both the K_m and K_i values are approximately identical to their K_s values. Therefore, in this report, K_i and K_m are used interchangeably and treated as approximate dissociation constants (K_s) of the Michaelis complexes.

With one exception, Ala substitutions in the octapeptide did not significantly change the K_m values. These results suggest that these peptides have similar affinities for renin in the ground state, as does the original peptide. The only change in K_m was observed with Ala substitution at the P1′ site. In this case, the increased K_m could result from a less favorable interaction between this peptide and renin. Alternatively, it could result indirectly from the more favorable solvation of the Ala-substituted peptide. However, this latter possibility is unlikely because we did not observe the same effect for an identical substitution of Leu by Ala at the P1 site. Furthermore, the study using additional amino acids to replace the P1′ Leu revealed that amino acids with large hydrophobic side chains have lower K_m values, suggesting that the S1′ site is relatively large and hydrophobic. This finding suggests that the K_m increase upon Ala substitution at the P1′ site is due to the less favorable interaction of the smaller Ala side chain with renin. This result indicates that, in addition to its important role in transition-state interactions, the P1′ residue also contributes to the ground-state interactions.

In an enzymatic reaction, the k_{cat} value reflects the energy barrier in proceeding from the Michaelis complex to the transition state, while the k_{cat}/K_m value reflects the transition-state energy barrier for the overall reaction starting from free enzyme and free substrate. Ala substitutions at the three crucial sites had dramatic effects on the k_{cat} and k_{cat}/K_m values. This observation suggests that these residues contribute to important interactions in the transition-state enzyme−substrate complex. These results are consistent with earlier studies that showed that hydrolyses of various tripeptide and tetrapeptide substrates by pepsin had values of k_{cat} that varied by 3 orders of magnitude and K_m values that changed by a factor of less than 4 (Inouye & Fruton, 1967; Sachdev & Fruton, 1970). Our results suggest that the stringent substrate specificity of renin is mostly achieved through the discrimination of substrates in the transition state. This provides another example demonstrating that the specificity of an enzymatic reaction is often manifested in the turnover number of the enzyme−substrate complex, with little change in the binding constants of the substrates (Jencks, 1975).

The observed enhancement of activity upon Ala substitution at the P2′ site is unexpected because renin is highly specific. However, this effect was observed with a synthetic peptide. It will be interesting to see whether the same enhancement can be detected with a similar substitution in the natural substrate, angiotensinogen. If so, it will be intriguing to know why the natural substrate of this highly specific enzyme is not the best substrate. *A priori*, one would expect that evolutionary selection should have optimized the interactions between a highly specific enzyme and its substrate. Since we used porcine renin in this study, it will also be interesting to see whether a similar activity enhancement can be detected with a similar substitution in the peptide substrate of human renin. If this is the case, one may take advantage of this fact in the design of renin inhibitors.

Renin is known to be species specific (Tewksbury et al., 1979). This specificity has been shown to result from different amino acid residues on the C-terminal side of the cleavage site of angiotensinogens from different species (Burton & Quinn, 1988). For example, human angiotensinogen is a poor substrate of porcine renin. The octapeptide corresponding to human angiotensinogen is H-P-F-H-L-V-I-H, whereas that of porcine angiotensinogen is H-P-F-H-L-L-V-Y. Ile substitution for the P2′ Val in the porcine substrate did not change its activity (Table 3; peptide 0 vs P2′ Ile). In contrast, replacement of the P1′ Leu with Val resulted in a 78-fold decrease in activity (Table 2; peptide 0

vs P1′ Val). Therefore, the P1′ residue plays a more important role than the P2′ residue in determining the species specificity of porcine renin, suggesting that the species specificities of renins may be mainly due to the differing P1′ residues in the angiotensinogens from different species.

The peptide with Phe replacing both Leu residues at the P1 and P1′ sites is an inhibitor of human renin (Poulsen et al., 1976; Burton et al., 1980; Cody et al., 1980). Phe substitution at the P1 site lowered the K_m value from 46.9 to 11.4 μM, but lowered the k_{cat} value only slightly. On the other hand, Phe substitution at the P1′ site resulted in a peptide with no significant change in K_m, but the k_{cat} value of the resultant peptide was decreased by a factor of 5. Combination of these two effects gives the double-Phe-substituted peptide a 4 times better affinity and a 7 times lower turnover rate. Therefore, this peptide can function as a moderate inhibitor when renin activity is assayed with other substrates (e.g., angiotensinogen).

ACKNOWLEDGMENT

We are grateful to Dr. Michael Gelb for the difluorostatone-containing renin inhibitor. We also thank Dr. Henry Strobel and Jane Li for comments and suggestions.

REFERENCES

Akahane, K., Umeyama, H., Nakagawa, S., Moriguchi, I., Hirose, S., Iizuka, K., & Murakami, K. (1985) *Hypertension 7*, 3−12.

Barany, G., & Merrifield, R. B. (1979) in *The Peptides* (Gross, E., & Meienhofer, J., Eds.) p 1, Academic Press, New York.

Blundell, T. L., Sibanda, B. L., & Pearl, L. H. (1983) *Nature (London) 304*, 273−275.

Boger, J., Payne, L. S., Perlow, D. S., Lohr, N. S., Poe, M., Alaine, E. H., Ulm., S. H., Schorn, T. W., LaMont, B. I., Lin, T.-Y., Kawai, M., Rich, D. H., & Veber, D. F. (1985) *J. Med. Chem. 28*, 1779−90.

Burton, J., & Quinn, T. (1988) *Biochim. Biophys. Acta 952*, 8−12.

Burton, J., Cody, R. J., Herd, J. A., & Haber, E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5476−5479.

Carlson, W., Karplus, M., & Haber, E. (1985) *Hypertension 7*, 13−26.

Cody, R. J., Burton, J., Evin, G., Poulsen, K., Herd, J. A., & Haber, E. (1980) *Biochem. Biophys. Res. Commun. 97*, 230−235.

Cumin, F., Le-Nguyen, D., Castro, B., Menard, J., & Corrol, P. (1987) *Biochim. Biophys. Acta 913*, 10−19.

Dealwis, C. G., Frazao, C., Badasso, M., Cooper, J. B., Tickle, I. J., Driessen, H., Blundell, T. L., Murakami, K., Miyazaki, H., Sueiras-Diaz, J., Jones, D. M., & Szelke, M. (1994) *J. Mol. Biol. 236*, 342−360.

Dhanaraj, V., Dealwis, C. G., Frazao, C., Badasso, M., Sibanda, B. L., Tickle, I. J., Cooper, J. B., Driessen, H. P. C., Newman, M., Aguilar, C., Wood, S. P., Blundell, T. L., Hobart, P. M., Geoghegan, K. F., Ammirati, M. J., Danley, D. E., O'Connor, B. A., & Hoover, D. J. (1992) *Nature 357*, 466−472.

Doherty, A. M., Kaltenbronn, J. S., Hudspeth, J. P., Repine, J. T., Roark, W. H., Sirca, I., Tinney, F., J., Connoly, C. J., Hodges, J. C., Taylor, M. D., Batley, B. L., Ryan, M. J., Essenburg, A. D., Rapundalo, S. T., Weishaar, R. E., Humblet, C., & Lunney, E. (1991) *J. Med. Chem. 34*, 1258−1271.

Evin, G., Castro, B., Devin, J., Menard, J., & Corvol, P. (1983) Peptide: Structure and Function, *Proceedings of the 8th American Peptide Symposium* (Hruby, V., & Rich, D., Eds.) pp 583−586, Pierce Chemical Co., Rockford, IL.

Fearon, K., Spaltenstein, A., Hopkins, P. B., & Gelb, M. H. (1987) *J. Med. Chem. 30*, 1617−1622.

Fruton, J. S. (1970) *Adv. Enzymol. Relat. Areas Mol. Biol. 33*, 401−433.

Galen, F. X., Devaux, C., Grogg, P., Menard, J., & Corvol, P. (1978) *Biochim. Biophys. Acta 523*, 485−493.

Greenlee, W. J. (1990) *Med. Res. Rev. 10*, 173−236.

Hemmings, A. M., Foundling, S. I., Sibanda, B. L., Wood, S. P., Pearl, L. H., & Blundell, T. L. (1985) *Biochem. Soc. Trans. 13*, 1036−1041.

Hutchins, C., & Greer, J. (1991) *Crit. Rev. Biochem. Mol. Biol. 26* (1), 77−127.

Inagami, T. (1981) in *Biochemical Regulation of Blood Pressure*. (Soffer, R. L., Ed.) pp 39−71, Wiley, New York.

Inagami, T. (1989) *J. Hypertens. 7* (Suppl. 2), S3−S8.

Inagami, T., & Murakami, K. (1977) *J. Biol. Chem. 252*, 2978−2983.

Inagami, T., Murakami, K., Misono, K., Workman, R. J., Cohen, S., & Suketa, Y. (1978) in *Acid Proteases, Structure, Function and Biology*, (Tang, J., Ed.) pp 225−247, Plenum Press, New York..

Inagami, T., Murakami, K., Yokosawa, H., Matoba, T., Yokosawa, N., Takii, Y., & Misosno, Y. (1980) in *Enzymatic Release of Vasoactive Peptides* (Gross, F., & Vogel, G., Eds.) pp 7−30, Raven Press, New York.

Inouye, K., & Fruton, J. S. (1967) *Biochemistry 6*, 1765−1776.

Jencks, W. P. (1975) *Adv. Enzymol. 43*, 219−410.

Murakami, K., & Inagami, T. (1979) in *Kinnins II. Systemic Proteases and Cellular Function* (Fujii, S., Moriya, H., Suzuki, T., Eds.) pp 617−627, Plenum Press, New York.

Poulsen, K., Haber, E., & Burton, J. (1976) *Biochim. Biophys. Acta 452*, 533−537.

Rosenberg, S. H., Plattner, J. J., Woods, K. W., Stein, H. H., Marcotte, P. A., Cohen, J., & Perun, T. J. (1987) *J. Med. Chem. 30*, 1224−1228.

Sachdev, G. P., & Fruton, J. S. (1970) *Biochemistry 9*, 4465−4470.

Schechter, I., & Berger, A. (1968) *Biochem. Biophys. Res. Commun. 32*, 898−902.

Sibanda, B. L., Blundell, T. L., Hobart, P. M., Fogliano, M., Bindra, J. S., Sominy, B. W., & Chirgwin, J. M. (1984) *FEBS Lett. 174*, 102−111.

Sibanda, B. L., Hemmings, A. M., & Blundell, T. L. (1985) in *Aspartic Proteinases and Their Inhibitors* (Kostka, V., Ed.) pp 339−349, de Gruyter, Berlin.

Sielecki, A. R., Hayakawa, K., Fujinaka, M., Murphy, M. E. P., Fraser, M., Muir, A. K., Carilli, C. T., Lewicki, J. A., Baxter, J. D., & James, M. N. G. (1989) *Science 243*, 1346−1351.

Skeggs, L. T., Lentz, K. E., Kahn, J. R., & Hochstrasser, H. (1968) *J. Exp. Med. 128*, 13−34.

Stewart, J. M., & Young, J. D. (1984) *Solid Phase Peptide Synthesis*, 2nd ed., Pierce Chemical Co., Rockford, IL.

Tewksbury, D. A., Dart, R. A., & Travis, J. (1979) *Circulation 59/60* (Suppl. II), 132.

Thaisrivongs, S., Mao, B., Pals, D. T., Turner, S. R., & Koll, L. T. (1990) *J. Med. Chem. 33*, 1337−1343.

Wang, W.& Liang, T. C. (1994) *Biochem. Biophys. Res. Commun. 201*, 835−840.