A Potent Radiolabeled Human Renin Inhibitor, [^{3}H]SR42128: Enzymatic, Kinetic, and Binding Studies to Renin and Other Aspartic Proteases

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ABSTRACT: The in vitro binding of [^{3}H]SR42128 (Iva-Phe-Nle-Sta-Ala-Sta-Arg), a potent inhibitor of human renin activity, to purified human renin and a number of other aspartic proteases was examined. SR42128 was found to be a competitive inhibitor of human renin, with a K_i of 0.35 nM at pH 5.7 and 2.0 nM at pH 7.4; it was thus more effective at pH 5.7 than at pH 7.4. Scatchard analysis of the interaction binding of [^{3}H]SR42128 to human renin indicated that binding was reversible and saturable at both pH 5.7 and pH 7.4. There was a single class of binding sites, and the K_D was 0.9 nM at pH 5.7 and 1 nM at pH 7.4. The association rate was 10 times more rapid at pH 5.7 than at pH 7.4, but there was no difference between the rates of dissociation of the enzyme–inhibitor complex at the two pHs. The effect of pH on the binding of [^{3}H]SR42128 to human renin, cathepsin D, pepsin, and gastricsin was also examined over the pH range 3–8. All the aspartic proteases had a high affinity for the inhibitor at low pH. However, at pH 7.4, [^{3}H]SR42128 was bound only to human renin and to none of the other aspartic proteases. Competitive binding studies with [^{3}H]SR42128 and a number of other inhibitors on human renin or cathepsin D were used to examine the relationships between structure and activity in these systems. The study as a whole indicates that pH plays a major role in the binding of [^{3}H]SR42128 to aspartic proteases and that the nature of the inhibitor residue reacting with the renin S_2 subsites is of critical importance for the specificity of the renin–inhibitor interaction.

Renin (EC 3.4.23.15) belongs to the aspartic protease family, which includes pepsin, cathepsin D, and gastricsin, but renin is peculiar in that it is specific for angiotensinogen, a glycoprotein synthesized by the liver. The product of the enzymatic reaction is the decapeptide angiotensin I, which is, in turn, immediately cleaved by a peptidyldipeptide hydrolase (EC 3.4.15.1) to form angiotensin II. Angiotensin II (AII) is a potent vasoconstrictor peptide implicated in the regulation of blood pressure and electrolyte metabolism. Because the renin–angiotensinogen reaction is rate limiting on AII generation, renin is an important target in the design of new antihypertensive drugs. Several approaches in the inhibition of the renin–angiotensinogen reaction have been adopted, such as the use of specific monoclonal renin antibodies (Galen et al., 1984), peptides derived from the renin profragment sequence (Cumin et al., 1985), substrate analogues (Szelke et al., 1982; Burton et al., 1975), and statine-containing peptides (Boger et al., 1983, 1985; Guegan et al., 1986).

Pepstatin (Iva-Val-Val-Sta-Ala-Sta),[1] an aspartic protease inhibitor discovered by Umezawa et al. (1970), contains an unusual amino acid, statine (Sta) [4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic acid]. This central statine residue is supposed to mimic the transition-state analogue conformation that the substrate scissile bond adopts during the enzymatic reaction (Marciniszyn et al., 1976). Statine-containing peptides could also be considered as collected-substrate inhibitors of acidic proteases, as described by Rich et al. (1985). Renin is competitively inhibited by pepstatin with K_i values varying from 10^{-5} to 10^{-7} M, according to species (Boger et al., 1983). Among the several compounds synthesized to increase the affinity for renin and to provide a stronger specificity toward this enzyme, a recent approach involved retention of the central statine residue, which corresponds to the dipeptide containing the scissile bonds Leu-Leu or Leu-Val of pig and human angiotensinogen, respectively. A better binding to the renin binding subsites S_4–S'_3 (Sibanda et al., 1984) was obtained by modifying the amino acids surrounding the central statine. Several compounds have been synthesized by our group and have been recently reported (Guegan et al., 1986). Among them, SR42128 (Iva-Phe-Nle-Sta-Ala-Sta) was obtained by replacing the dipeptide Val-Val of the pepstatin sequence by the dipeptide Phe-Nle. SR42128 was 1000-fold more potent than pepstatin in inhibiting human plasma renin activity (PRA) at physiological pH (De Claviere et al., 1985).

In this last study, as in most studies reported so far in the literature, human renin inhibition has been studied at pH 7.4 by using impure enzymatic systems such as plasma renin acting on endogenous plasma angiotensinogen (plasma renin activity). In this work, the inhibition mechanism of SR42128 was studied in a pure renin assay using highly purified human renin and a synthetic substrate, corresponding to the fourteen NH_2-terminal amino acids of human angiotensinogen.

Radiolabeled enzyme inhibitors of high specific activity are useful tools for the direct measurement of association and dissociation kinetic constants, especially for very tight binding inhibitors where classic enzymatic reactions cannot be applied. No studies have yet been reported on the use of high-affinity radioligands for renin. The more recent binding studies reported on aspartic proteases have been performed by using a tritiated pepstatin analogue [^{3}H]Gly-pepstatin on human cathepsin D (Knight & Barrett, 1976) or by using a radioiodinated pepstatin derivative on porcine pepsin (Workman & Burkitt, 1979). Because SR42128 had a low inhibitory constant for renin, it was decided to tritiate this compound

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[1] Abbreviations: Sta, statine or 4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic acid; Iva, isovaleryl; ELISA, enzyme-linked immunosorbent assay; Boc, *tert*-butyloxycarbonyl; ACHPA, 4(*S*)-amino-3(*S*)-hydroxy-5-cyclohexylpentanoic acid.

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Table I: Amino Acid Sequences of Unlabeled Renin Inhibitors Used in Competition Studies

compd	renin subsites[a]										ref
	S_6	S_5	S_4	S_3	S_2	S_1	S'_1	S'_2	S'_3	S'_4	
1			Iva-	Val-	Val-		Sta-	Ala-	Sta-	OH	*b*
2			Iva-	Phe-	Nle-		Sta-	Ala-	Sta-	OH	*b*
3			Boc-	Phe-	Nle-		Sta-	Ala-	Sta-	OH	*c*
4			Boc-	Phe-	His-		Sta-	Ala-	Sta-	OH	*b*
5			Iva-	Phe-	His-		Sta-	Ala-	Sta-	OH	*c*
6			Boc-	Phe-	His-		ACHPA-	Leu-	PMA		*d*
7	Iva-	His-	Pro-	Phe-	His-		Sta-	Ile-	Phe-	NH_2	*e*
8	Pro-	His-	Pro-	Phe-	His-	Leu^R	Val-	Ile-	His-	Lys-OH	*f*

[a] Abbreviations: Iva, isovaleryl; R, reduced bond $-CH_2-NH$; ACHPA, 4(*S*)-amino-3(*S*)-hydroxy-5-cyclohexylpentanoic acid; Nle, norleucine; Sta, statine or 4(*S*)-amino-3(*S*)-hydroxy-6-methylheptanoic acid; Boc, *tert*-butyloxycarbonyl; PMA, (2-pyridylmethyl)amide. [b] Guegan et al. (1986). [c] Present study. [d] Breipohl et al. (1984). [e] Boger et al. (1983). [f] Szelke et al. (1982).

at high specific activity and to study its direct interaction with various aspartic proteases such as renin, pepsin, gastricsin, and cathepsin D. The effect of pH on [^{3}H]SR42128 binding on this class of proteins was studied. Finally, [^{3}H]SR42128 was used as a reference compound in competition studies in which several unlabeled compounds were compared under different experimental conditions without being limited by the pH range of substrate hydrolysis or by the use of semipurified enzyme and/or substrate preparations.

Experimental Procedures

Materials

[^{3}H]SR42128 (Iva-Phe*-Nle-Sta-Ala-Sta-Arg) was obtained by catalytic reduction on palladium-activated charcoal of the *p*-iodophenyl derivative of SR42128. One tritium residue was incorporated per molecule of SR42128, giving a specific activity of 30 Ci/mmol. The radiolabeled compound gave a single spot on thin-layer silica gel chromatography using $CHCl_3$/MeOH/CH_3COOH (90/10/1) as eluent (R_f 0.32). Renin inhibitors **1**–**7** (Table I) were synthesized by Sanofi Research Center (Montpellier, France). Compound **8** (H142) was a generous gift from Dr. Szelke (London, U.K.). Dextran T70 and charcoal were purchased from Pharmacia. Scintillation solution for counting was from Packard (Pico-Fluor 15), and all other chemicals and solvents were reagent grade from Merck.

Highly purified active human renin (specific activity 310 Goldblatt units/mg of protein) was obtained by using a monoclonal antibody and an immunoaffinity column (Galen et al., 1984). Pure human pepsin was a generous gift from Dr. J. Tang (Oklahoma City, OK). Pure human cathepsin D and gastricsin were gifts from Dr. A. Barrett (Cambridge, U.K.). Pure human tetradecapeptide substrate was purchased from Bachem (Bubendorf, Switzerland).

Methods

Inhibition of Human Plasma Renin Activity (PRA). The inhibitory potencies of compounds **3** and **5**–**8** (Table I) on human PRA were studied at pH 7.4 as described by Guegan et al. (1986). Results are expressed as IC_{50} values, which correspond to the molar concentration of inhibitor causing 50% inhibition of PRA.

Enzymatic Kinetic Studies. (1) Time Course of Angiotensin I Generation. Human renin (1.25 nM) and human synthetic substrate (2.7 μM) were incubated in the absence or presence (1 mM) of SR42128 for various times from 5 to 120 min in 0.5 M citrate–phosphate buffer containing 1 mg/mL bovine serum albumin at pH 5.7. In the presence of SR42128 the enzymatic reaction was started either after a 60-min preincubation of SR42128 with human renin or directly by addition of the substrate. Aliquots were taken at different times, the reaction was stopped by chilling in an ice bath, and the amount of angiotensin I (AI) generated was measured by radioimmunoassay as described by Mênard and Catt (1972).

(2) Determination of Kinetic Constants K_i. Human renin (0.09 nM) and pure human synthetic tetradecapeptide substrate (Asp_1–Asn_{14}) (1.35–10.8 μM) were incubated for 60 min at 37 °C in the presence or absence of 10^{-10}–10^{-8} M SR42128. Kinetic assays were performed in a 0.5 M citrate–phosphate buffer containing 1 mg/mL bovine serum albumin either at pH 5.7 (buffer A) or at 7.4 (buffer B), in a total incubation volume of 0.5 mL. AI produced was measured as described above. Data were analyzed by using a Lineweaver and Burk (1934) plot to determine the inhibition mode and a Dixon (1983) plot to estimate the K_i values. All lines were fitted with a least-squares method by using computer programs.

Binding Assays. A dextran-coated charcoal method was selected to separate bound from free [^{3}H]SR42128. The charcoal was prepared in 5 mg/mL bovin serum albumin to avoid nonspecific absorption. Assay samples of 400 μL were incubated for 10 min with 400 μL of dextran-coated charcoal (0.25% dextran, 1.25% charcoal) and centrifuged at 4000*g* for 15 min. The supernatants were decanted into counting vials, and the aqueous samples were counted after addition of 5 mL of Pico-Fluor 15 (Packard). It was verified that no significant dissociation of [^{3}H]SR42128 from renin occurred during the exposure to dextran-coated charcoal for 16 min. All human renin concentrations were checked by a renin ELISA (Menard et al., 1984). Human renin binding assays of [^{3}H]SR42128 were performed in buffer A and in buffer B. Human cathepsin D was assayed in 0.5 M citrate–phosphate buffer containing 1 mg/mL bovine serum albumin at pH 4.0 (buffer C). All experiments were performed at 20 °C.

Determination of Kinetic Association Parameters (k_1). Kinetic association constants (k_1) were determined at pH 5.7 by incubating [^{3}H]SR42128 (1.9 nM) with human renin (0.6 nM) in buffer A for various times between 1 and 180 min. For pH 7.4 studies, [^{3}H]SR42128 (1.3 nM) was incubated with human renin (0.6 nM) in buffer B for periods from 1 to 180 min. All incubations were done in parallel with controls containing 10^3-fold excess of unlabeled peptide, which was then subtracted from the total bound. Nonspecific binding never exceeded 5% of the total inhibitor concentration present in the assay. Details of the mathematical analysis of data are reported below.

Determination of Kinetic Dissociation Constants (k_{-1}). Kinetic dissociation constants (k_{-1}) were determined at pH 5.7 by incubating [^{3}H]SR42128 (6.0 nM) with human renin (0.3 nM) for 1 h in buffer A to reach the equilibrium. For pH 7.4 studies, [^{3}H]SR42128 (11.9 nM) was incubated with human renin (2.4 nM) in buffer B as described above. After

1 h, an excess of unlabeled SR42128 (10^{-6} M) was added to the incubation medium. Bound SR42128 was determined at various times from 1 to 40 min, as described above.

Equilibrium Saturation Studies. Increasing concentrations of [^{3}H]SR42128 (10^{-12}–10^{-7} M) were incubated for 1 h at 20 °C with human renin in buffer A and in buffer B. Total radioactivity (T) was then counted, and bound peptide (B) was separated from unbound (U) as described above and counted. Unbound peptide was calculated as the difference between T and B. Binding parameters were determined by a computer method described by Claire et al. (1978), which uses several iterations to obtain the best fit curves for interaction of a ligand with one or more specific and nonspecific binding sites. Scatchard plots (Scatchard, 1949) were used to display the results.

Competition Studies Performed with Human Renin and Cathepsin D. Human renin (0.66 nM) and [^{3}H]SR42128 (1 nM) were incubated in buffer B either alone or in the presence of increasing concentrations of unlabeled competitors. Human cathepsin D (10^{-8} M) and [^{3}H]SR42128 (3.8 nM) were incubated in buffer C either alone or in the presence of increasing concentrations of unlabeled inhibitors. For the two assays, after 1 h of incubation, bound and free [^{3}H]SR42128 were separated as described above. Data were plotted by using a semilogarithmic representation. Each inhibitor was characterized by its IC_{50} value, corresponding to the concentration of unlabeled inhibitor able to displace 50% of the bound [^{3}H]SR42128.

Effect of pH on [^{3}H]SR42128 Binding to Various Aspartic Proteases. [^{3}H]SR42128 (15 nM) was incubated for 1 h with equimolar concentrations (1.7 nM) of human renin, pepsin, cathepsin D, or gastricsin in 0.5 M phosphate buffer containing 1 mg/mL of bovine serum albumin at pH values varying from 3.5 to 8. The bound fraction was estimated as described above.

Binding Data Analysis. It was assumed that the binding reaction was as simple bimolecular process that obeyed the law of mass action as predicted for a uniform population of noninteracting binding sites according to

$$L + R \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} LR$$

where R represents the renin binding site, L is [^{3}H]SR42128, LR is the bimolecular complex formed, and k_1 and k_{-1} represent the forward and reverse rate constants, respectively. $(LR)_e$ represents the total molar concentration of ligand–receptor complex found at steady state.

The association rate constant k_1 was determined from the reversible second-order rate equation (Weiland & Molinoff, 1981):

$$\ln\left[\frac{(LR)_e[(L)_t - (LR)(LR)_e/(R)_t]}{(L)_t[(LR)_e - (LR)]}\right] = k_1[[(L)_t(R)_t]/(LR)_e - (LR)_e]t \quad (1)$$

where $(L)_t$ and $(R)_t$ represent the total molar concentrations of ligands and renin in the assay, respectively. $(R)_t$ was independently determined by ELISA, as described previously. The dissociation rate constant for [^{3}H]SR42128, k_{-1}, was estimated from a weighted least-squares regression analysis of the first-order plot of logarithmically transformed data, according to

$$\ln [(LR)/(LR)_e] = k_{-1}t \quad (2)$$

The plot of ln $[(LR)/(LR)_e]$ vs time gave a straight line with a slope equal to the first-order dissociation rate constant (k_{-1}). The ratio of forward and reverse rate constants was used to calculate the dissociation constant (K_D) which is independent of equilibrium methods according to the relationship $K_D = k_{-1}/k_1$.

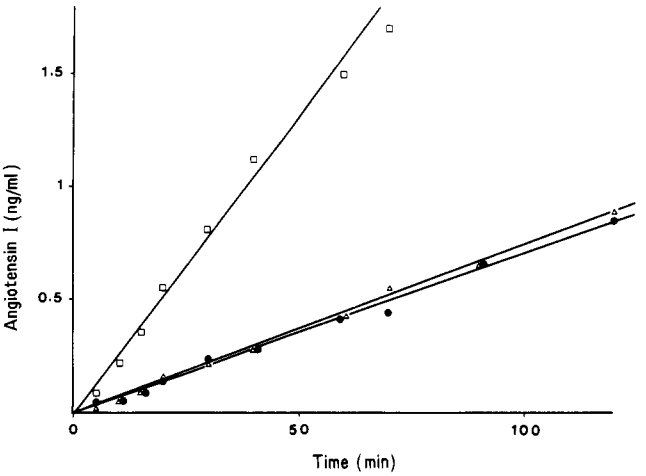

FIGURE 1: Time course of human tetradecapeptide (Asp_1–Asn_{14}) hydrolysis by human renin in the absence of SR42128 (□), after a preincubation of SR42128 with renin (●), or without preincubation (△) prior to start of the enzymatic reaction by addition of substrate.

Table II: Effect of pH on Kinetic and Equilibrium Constants Characterizing the Interaction between Renin and SR42128

pH	K_i (nM)	k_1 (10^9 M^{-1} min^{-1})	k_{-1} (min^{-1})	k_{-1}/k_1	K_D (nM)
5.7	0.35	0.1	0.03	0.3	0.51
7.4	2.0	0.01	0.03	3	7

RESULTS

Inhibition Studies. The inhibitory effect of SR42128 on renin was first studied by using a standard kinetic approach. The same rate of angiotensin I generation in the presence of inhibitor was observed whether the enzymatic reaction was started by addition of the substrate or enzyme (Figure 1). SR42128 appeared to be a simple competitive inhibitor of the hydrolysis of human tetradecapeptide Asp_1–Asn_{14} as determined by Lineweaver–Burk plot analysis at pH 7.4 (Figure 2A) and 5.7 (results not shown). The efficiency of this inhibitor was expressed by its K_i value, which represents the dissociation constant for inhibitor and free enzyme. It was determined by a plot of $1/V$ vs [I] at various substrate concentrations, yielding straight lines that intersect at $[I] = -K_i$ (Dixon, 1953) (Figure 2B). K_i values were 0.35 and 2 nM at pH 5.7 and 7.4, respectively.

[^{3}H]SR42128 Interactions with Human Renin. The interaction of human renin with [^{3}H]SR42128 was studied quantitatively and directly by using equilibrium and kinetic experiments. [^{3}H]SR42128 bound human renin reversibly, and binding sites were saturable at high concentrations of peptide. Figure 3 shows that equilibrium at nonsaturating levels of [^{3}H]SR42128 was obtained after 60 min at pH 5.7 and 7.4 and did not vary after 12 h (data not shown). The dissociation of [^{3}H]SR42128 from renin at pH 5.7 and 7.4 is shown in Figure 3. Association and dissociation binding rates of [^{3}H]SR42128 to human renin were monophasic and could be used to calculate the association (k_1) and dissociation (k_{-1}) kinetic parameters of the equilibrium, as described under Experimental Procedures (Table II). At pH 5.7, [^{3}H]-SR42128 was bound to human renin 10-fold more rapidly that at pH 7.4 whereas the k_{-1} values did not change.

Figure 4 shows a Scatchard plot of [^{3}H]SR42128 binding to human renin at pH 5.7. A similar experiment was con-

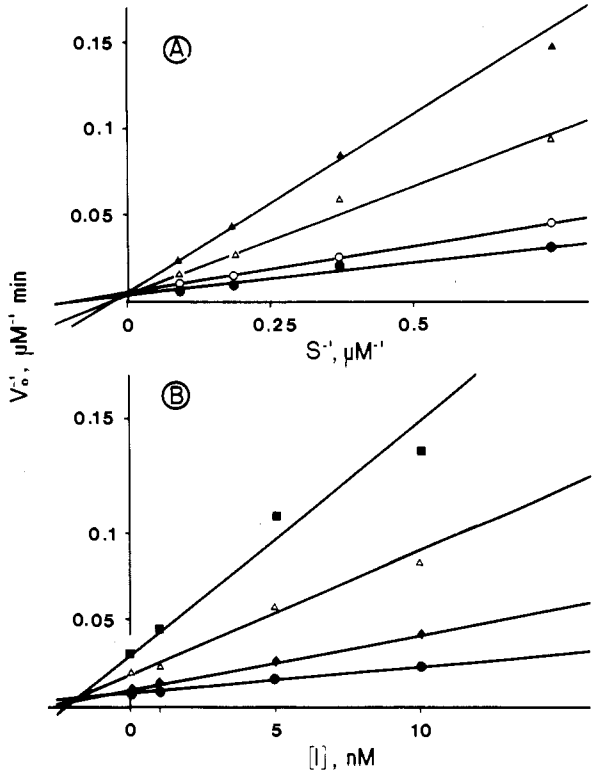

FIGURE 2: Lineweaver–Burk (A) and Dixon (B) plots of the inhibition of human renin by SR42128. Assays were performed at 37 °C, in 0.5 M citrate–phosphate buffer, pH 7.4. (A) Inhibitor concentrations: (●) 0.0, (○) 1.0, (△) 5.0, and (▲) 10.0 nM. (B) Substrate concentrations: (■) 1.35, (△) 2.7, (♦) 5.4, and (●) 10.8 μM.

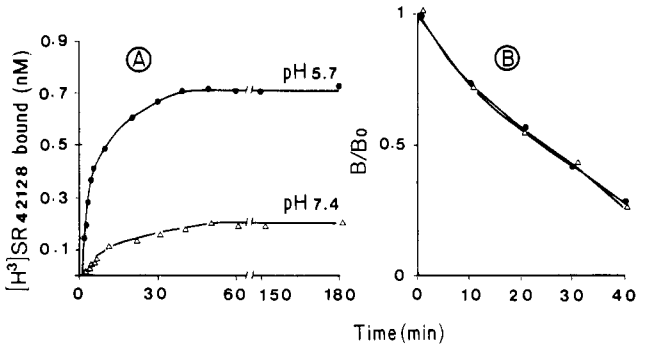

FIGURE 3: Effect of pH on rates of association and dissociation of [^{3}H]SR42128 from pure human renin at pH 5.7 and 7.4. (A) [^{3}H]SR42128 was incubated with pure human renin (0.6 nM) for the indicated times at 20 °C. Bound and free fractions were separated, and the association kinetic constants (k_1) were calculated as described under Experimental Procedures and reported in Table II. Each time point was the mean of triplicates. (B) After the steady state between [^{3}H]SR42128 and human renin had been reached, an excess of unlabeled SR42128 (10^{-6} M) was added to the incubation medium. The changes in bound [^{3}H]SR42128 as followed with time and dissociation kinetic constants (k_{-1}) were calculated as described under Experimental Procedures. Each time point was the mean of triplicates. Values are summarized in Table II.

ducted at pH 7.4 (results not shown). When the computer program described above was used, the data fitted a single binding site model at both pHs. The dissociation constant (K_D) calculated by computer analysis was 14-fold higher at pH 7.4 (7 nM) than at pH 5.7. These values were in excellent agreement with those deduced from the ratio of dissociation and association kinetic constants (Table II). The maximal concentration of binding sites (B_{max}) was to 2.5 nM at pH 5.7 and 1.7 nM at pH 7.4, respectively. These results were in good agreement with those obtained by direct measurement of renin concentration by ELISA (2.35 nM at pH 5.7 and 1.85 nM

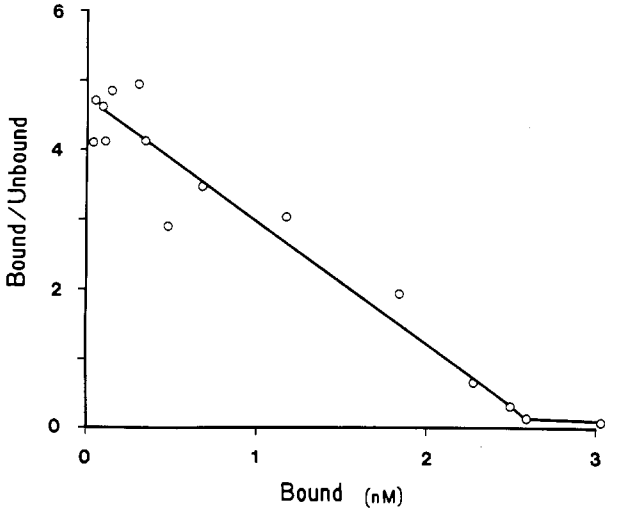

FIGURE 4: Scatchard plot of [^{3}H]SR42128 binding to human renin at pH 5.7. Human renin (2.35 nM) was equilibrated for 1 h with varying concentrations of [^{3}H]SR42128. Bound and free fractions of [^{3}H]SR42128 were separated as described under Experimental Procedures. The value of K_D was 0.51 nM, and that of B_{max} was 2.5 nM.

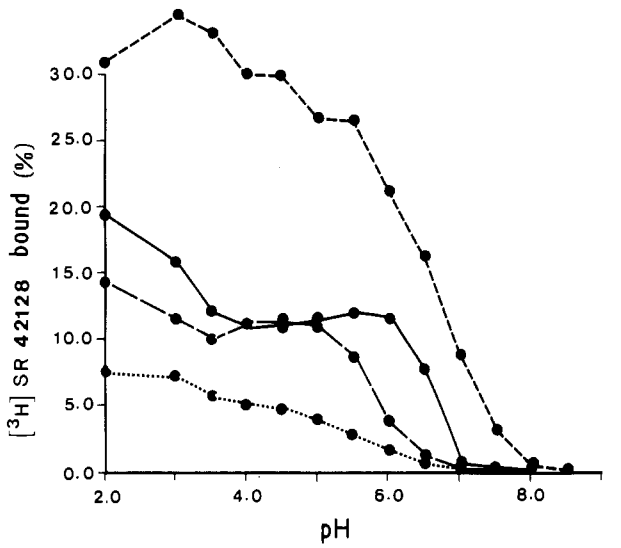

FIGURE 5: Effects of pH on the specific binding of [^{3}H]SR42128 to human acidic proteases. Human renin (●---●), cathepsin D (●···●), pepsin (●—●), and gastricsin (●–·–●) at similar concentrations (1.7 nM) were incubated separately with [^{3}H]SR42128 (15 nM) for 1 h at the indicated pH values. [^{3}H]SR42128 specifically bound to each acidic protease was determined at each pH value.

at pH 7.4). This suggests the existence of one binding site for [^{3}H]SR42128 per molecule of renin.

Effect of pH on [^{3}H]SR42128 Binding to Renin and Other Aspartic Proteases. The influence of pH on the specific binding of [^{3}H]SR42128 was investigated by incubating [^{3}H]SR42128 with equimolar concentrations of various human aspartic proteases, in 0.5 M phosphate buffer. A large decrease in the specific binding of [^{3}H]SR42128 to renin, cathepsin D, pepsin, and gastricsin was measured when the pH was increased from 3 to 8 (Figure 5). All aspartic proteases, including renin, were able to bind [^{3}H]SR42128, with maximum binding at low pH. However, [^{3}H]SR42128 was preferentially bound to renin at neutral pH, indicating a higher specificity for renin at this pH.

Competitive Binding of [^{3}H]SR42128 and Unlabeled Inhibitors to Human Renin and Human Cathepsin D. Figure 6A shows the competitive binding curves obtained at pH 7.4 with human renin, and Figure 6B shows the curves obtained

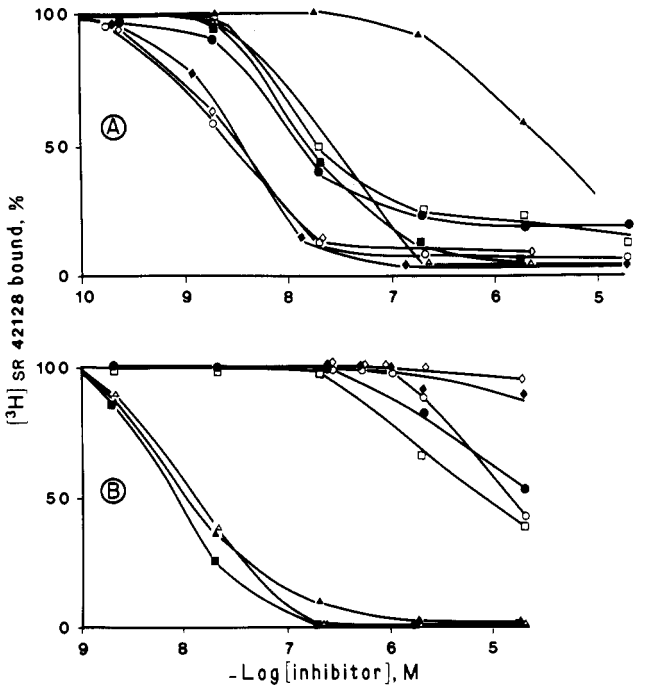

FIGURE 6: Competition experiments between [^{3}H]SR42128 and various unlabeled renin inhibitors (Table I). The binding of [^{3}H]-SR42128 to (A) human renin (1 nM) at pH 7.4 or (B) human cathepsin D (10 nM) at pH 4.0 was determined in the presence of the indicated concentration of unlabeled inhibitor. Curves represent the percent of specifically bound [^{3}H]SR42128. The inhibitor sequences summarized in Table I are **1** (▲), **2** (■), **3** (△), **4** (●), **5** (□), **6** (○), **7** (♦), and **8** (◇).

Table III: Effect of Renin Inhibitors on [^{3}H]SR42128 Binding and Renin Enzymatic Activity [IC_{50} (nM)]

	[^{3}H]SR42128 binding		PRA
compd	renin, pH 7.4	cathepsin D, pH 4.0	inhibition, pH 7.4
1	3000	<10	14000[a]
2	15	<10	28[a]
3	26	<10	46
4	13	>10^4	45[a]
5	20	10^4	64
6	3	>10^4	4
7	3.5	>10^4	1.3
8	3.5	>10^4	10

[a] Guegan et al. (1986).

at pH 4.0 with human cathepsin D. The structures of the unlabeled renin inhibitors and of the corresponding renin subsite localizations are reported in Table I. Pepstatin (compound **1**) was used as a reference compound, and its IC_{50} (amount of unlabeled compound displacing 50% of the specifically bound [^{3}H]SR42128) was 3 μM (Table III). Compounds **2–5** had similar affinities in the human renin assay, with IC_{50} values in the 10^{-8} M range. Compounds **6–8** were the most potent renin inhibitors studied, with IC_{50} values in the nanomolar range. In the cathepsin D assay, compounds **4–8** had IC_{50} values higher than 10^{-5} M. Pepstatin and compounds **2** and **3** had IC_{50} values equal to 10^{-8} M. These values were underestimated because, as described by Jacobs et al. (1975), IC_{50} values found in competition studies are limited by the sum of enzyme and radioligand concentrations present in the assay.

Discussion

This study reports, for the first time, the interaction of a highly potent radiolabeled renin inhibitor with renin and other members of the aspartic enzyme family. SR42128 was selected from the compounds synthesized by our group because of its high affinity and selectivity for human renin compared to renin from other species (Guegan et al., 1986). SR42128 was previously reported to be a potent inhibitor of human plasma renin activity (PRA), exhibiting IC_{50} values equal to 28 nM at pH 7.4 (De Clavière et al., 1984). However this measurement, like most renin assays for the screening of renin inhibitors, was performed with an impure system, whereas the use of the present defined renin and substrate eliminates the effects of putative plasma activator (Sambhi et al., 1975), plasma inhibitors (Kotchen et al., 1975), nonspecific binding to plasma proteins, and enzymatic degradation which could occur in plasma assays. The mechanism of inhibition of pepsin by pepstatin has been reported to be time-dependent by Rich and Sun (1980). In such a case, a preincubation is required to reach the equilibrium between enzyme and inhibitor prior to the start of the enzymatic reaction by addition of substrate. Such experiments cannot be performed by using plasma assays where renin and angiotensinogen are already in contact. We have compared the rates of angiotensin I generation with or without a 60-min preincubation of renin with SR42128 before the enzymatic reaction was started by the addition of the substrate. The steady velocity was followed from 5 to 120 min. No differences in the rate of angiotensin I generation was noted, and therefore no preincubation of the enzyme with the inhibitor was performed in subsequent experiments.

In the present study, the analysis of SR42128 inhibition using a pure renin assay showed that this statine-containing peptide behaved as a competitive renin inhibitor. The K_i value obtained in the pure assay was equal to 2 nM at physiological pH, which was much lower than K_i value calculated by using the equation described by Cheng and Prusoff (1973), $K_i = IC_{50}/(1 + S/K_m)$, where the IC_{50} values have been determined by PRA assays. This discrepancy may be due to the hydrophobicity of this compound and therefore its adsorption to plasma components. These nonspecific interactions likely account for the different potencies reported for renin inhibitors in pure systems and in plasma assays (Boger et al., 1985).

Similar values for dissociation (K_D) and inhibition constants (K_i) have been found, suggesting that they characterize the same equilibrium. The good correspondence found between the maximal binding site concentration (B_{max}) and the direct measurement of renin concentration by an ELISA method indicated a single binding site per molecule of renin. Monophasic association and dissociation curves were found at pH 5.7 and 7.4, showing that [^{3}H]SR42128 was probably bound to human renin by a simple bimolecular mechanism. There was no evidence for an additional isomerization step similar to that reported for pepstatin and pepsin (Rich et al., 1980). This was supported by the monophasic dissociation and the ratio of association and dissociation rate constants close to the K_D value obtained by equilibrium studies.

It was previously reported that compounds analogous to SR42128 showed a marked increase in inhibitory potency when studied at pH 6.0 compared to pH 7.4 in PRA assays (De Clavière et al., 1985). The present study shows also that pH plays an important role in the inhibition since the renin inhibition parameter (K_i) obtained for SR42128 at pH 7.4 was 5.7-fold higher than that at pH 5.7. A similar pH effect was observed for the direct SR42128 binding at pH 5.7 and 7.4. The difference in binding was mainly due to the rate of association of the inhibitor since these constants (k_1) were 2–3 orders of magnitude smaller than the values obtained for diffusion-limited addition mechanism of small molecules to

enzymes (Hammes & Schimmel, 1970) (6×10^{10}–6×10^{12} M^{-1} min^{-1}). This suggests that the formation of the enzyme–inhibitor complex needs more than a simple encounter between the two molecules. The molecular mechanism(s) involved by this difference of pH can only be speculative. However, in this pH range, a histidyl residue in its protonated form or a basic residue could be involved either in stabilizing the enzyme–inhibitor complex or in facilitating the accessibility of the various subsites present in the cleft. In addition, the renin active site may be less accessible at physiological pH than at pH 5.7 because the flap region of renin overlaps the renin active site (Blundell et al., 1983) and, therefore, may interact with inhibitors or substrates, resulting in a decrease in the rate of association. No pH-dependent difference was found in the rate of dissociation, suggesting that the enzyme-inhibitor complex adopts a conformation that is independent of pH. A similar pH effect has been reported for human cathepsin D, with a tritiated derivative of pepstatin, [^{3}H]Gly-pepstatin. The association constant (K_a) determined by equilibrium dialysis decreased 10^3-fold when the pH values was increased from 5.0 to 6.4 (Knight & Barrett, 1976); this was attributed to the loss of three protons from human cathepsin D.

Binding of [^{3}H]SR42128 to human renin, human pepsin, gastricsin, and cathepsin D was investigated at various pH values. All the acidic proteases, including renin, showed higher affinity for SR42128 at acidic pH. No strong specificity of [^{3}H]SR42128 binding to human renin was observed except at physiological pH, where the radioligand binds to human renin but not to the other aspartic proteases.

The relative affinity of a number of renin inhibitors was compared in the competition studies performed on the binding of [^{3}H]SR42128 bound to human renin and to cathepsin D. Human renin probably has a catalytic site similar to those of other well-known aspartic proteases, such as penicillopepsin (James et al., 1982) and *Rhizopus chinensis* (Bott et al., 1982). Compounds **2**–**5** which only differ by the substitution of an Iva residue for a Boc residue, showed similar IC_{50} values in both binding and enzymatic studies. This corresponds to the S_4 renin subsite, which is occupied by a proline residue in the angiotensinogen molecule. Its spatial dimension corresponds approximately to that of Iva and Boc residues. A histidine and a phenylalanine residue interact with S_2 and S_3 renin subsites, respectively, in the natural substrate. This accounts for the large increase in renin binding efficiency shown by all inhibitors in which the dipeptide Val-Val present in pepstatin is replaced by the dipeptides Phe-Nle or Phe-His. The most interesting results are those concerning the human renin specificity. Compounds **2** and **3** possess a norleucine residue which interacts with the S_2 renin subsite. These showed a high binding potency toward both human renin and cathepsin D. At the other extreme, compounds **4**–**8**, which possess a histidine residue at the same position, were highly specific for human renin because they bound cathepsin D much less efficiently. This showed the importance of the interaction of a histidine residue with the renin S_2 subsite for determining human renin specificity. In addition, Guegan et al. (1986) reported that compounds **4** and **5** were stronger inhibitors of human renin than of other mammalian renins, suggesting that the histidine interaction in subsite 2 increase species specificity of the inhibitor for human renin. The two compounds **7** and **8** previously studied by Boger et al. (1983) possess long sequences interacting within the renin subsites S_6–S_3. They showed a high binding affinity toward human renin and a low affinity for human cathepsin D. A 10-fold higher renin inhibitory potency has been measured with compound **7** than with compound **8**. Compound **6** is similar to compound **4**, but it exhibited a higher renin binding potency which might be due to the presence of a statine derivative, ACHPA, which interacts with the S_1–S'_1 subsites. Enzymatic comparisons between statine- and ACHPA-containing peptides have been previously performed by Boger et al. (1985), who showed that the presence of ACHPA increased the binding potency and the specificity toward human renin.

The potency of compounds **6**–**8** in the human renin assay and compounds **1**–**3** in the human cathepsin D assay might be underestimated. The IC_{50} values deduced from semilogarithmic plots of specifically bound labeled SR42128 are usually related to the K_d value of unlabeled competitors (Cheng & Prusoff, 1977) and in the same order of magnitude as them. In some cases, the IC_{50} value may be limited by the concentration of labeled ligand and/or the concentration of enzyme present in the assay medium (Jacobs et al., 1975). This explains why pepstatin in our human cathepsin D assay showed an IC_{50} value equal to 10^{-8} M, whereas Knight and Barrett (1976) reported a K_D value of 4.7×10^{-10} M at pH 3.5 for pepstatin. Nevertheless, competition studies can be extremely useful in direct comparisons of the relationships between structure, activity, and inhibitor specificity.

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Kinetics of the Inhibition of Human Renin by an Inhibitor Containing a Hydroxyethylene Dipeptide Isostere

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ABSTRACT: We have studied the inhibition of both human and hog renins by compound **1** [Boc-Pro-Phe-N^{α}-MeHis-Leuψ(CHOHCH$_2$)Val-Ile-(aminomethyl)pyridine] using kinetics. The inhibition of human renin was shown to be time dependent and followed a minimal two-step mechanism. A loosely bound EI complex was formed rapidly with a dissociation constant, K_I, of 12 nM. A second EI complex was slowly formed and was found to be 64-fold more strongly bound with an overall K_I* of 0.19 nM. The inhibition of human renin was shown to be competitive by both initial and final steady-state velocities. Compound **1** was also shown to be a competitive inhibitor of hog renin with a K_I of 12 nM, but no evidence for time-dependent inhibition was detected. The differences in overall K_I and inhibition kinetics may be a consequence of the similarities in structure between **1** and human angiotensinogen.

The aspartyl protease renin (EC 3.4.99.19) is secreted by specialized cells in the kidney into the bloodstream where it cleaves an N-terminal fragment from its sole substrate angiotensinogen to form the decapeptide angiotensin I. A C-terminal dipeptide is removed from angiotensin I by angiotensin-converting enzyme to generate the biologically active octapeptide angiotensin II. Angiotensin II causes vasoconstriction by binding to its receptor on the arterial walls, but it also stimulates the release of aldosterone from the adrenal glands. High aldosterone levels induce sodium and water retention, leading to an increase in blood pressure by a volume mechanism as well (Peach, 1977). The renin–angiotensin cascade has been shown to participate in both the maintenance of normal blood pressure (MacGregor et al., 1981) and certain forms of hypertension (Laragh, 1981).

The inhibition of renin is an attractive site for control of the cascade because renin has no other known physiological role. Potent renin inhibitors have been described that incorporate either a reduced peptide bond ψ(CH$_2$NH),[1] a hydroxyethylene bond ψ(CHOHCH$_2$), or the unusual amino acid statine[2] into peptides that contain portions of the angiotensinogen substrate sequence (Szelke et al., 1982, 1983; Boger et al., 1983). However, detailed investigations of the kinetics of inhibition for these compounds were not reported. Such

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[1] The abbreviation ψ[x], indicating that x replaces the amide –CONH– unit, has been defined by the IUPAC–IUB Joint Commission on Biochemical Nomenclature (1984).

[2] Abbreviations: statine, (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid; PMSF, phenylmethanesulfonyl fluoride; pNPGB, *p*-nitrophenyl *p*-guanidinobenzoate; RIA, radioimmunoassay; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Boc, *tert*-butyloxycarbonyl; Amp, 2-(aminomethyl)pyridine; Na$_2$EDTA, ethylenediaminetetraacetic acid disodium salt; BSA, bovine serum albumin; AI, angiotensin I; EI, enzyme–inhibitor complex.

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