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INTERACTION OF MOUSE SUBMAXILLARY GLAND RENIN WITH A STATINE-CONTAINING, SUBNANOMOLAR, COMPETITIVE INHIBITOR

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The interaction between mouse submaxillary gland renin and a statine-containing, iodinated substrate analog inhibitor was studied. The compound, **1** (Boc-His-Pro-Phe-(4-iodo)-Phe-Sta-Leu-Phe-NH_2, Sta = (3*S*,4*S*)-4-amino-3-hydroxy-6-methyl-heptanoic acid), a statine-containing analog of the renin substrate octapeptide, was a competitive inhibitor of cleavage of synthetic tetradecapeptide renin substrate by mouse submaxillary gland renin, with a K_i of 6.2×10^{-10} M (pH 7.2, 37°C). Titration of the partial quenching of the tryptophan fluorescence of the enzyme by **1** revealed tight binding with a dissociation constant less than 3 nM and a binding stoichiometry of one mole **1** per mole enzyme. The time course of tight binding of **1** to mouse renin appeared to be fast, with $k_{ON} \geqslant 1.3 \times 10^6\ s^{-1}\ M^{-1}$. The UV difference spectrum generated upon binding of **1** to mouse renin had two prominent features: a strong, broad band that had a minimum at 242 nm with $\Delta\varepsilon(242) = -19\,500\ cm^{-1}\ M^{-1}$, and a triplet of enhanced bands centered at 286 nm with $\Delta\varepsilon(286)$ about $+1100\ cm^{-1}\ M^{-1}$. The strong, broad, negative band was similar to the difference between the UV absorbance of **1** in methanol and in 0.1 M citrate phosphate pH 7.2. A structure-activity correlation for analogs of **1** showed some moieties of **1** that are important for potent inhibition of mouse renin. The inhibition data for these compounds versus human kidney renin suggested that the solution of the crystal structure of **1** bound to mouse renin will provide useful information for the design of inhibitors of human kidney renin.

KEYWORDS: Renin, statine, mouse renin, renin inhibitor.

ABBREVIATIONS: Abbreviations used: compound **1**, *N*-α-(*t*-butyloxycarbonyl)-L-histidinyl-L-prolyl-L-phenylalanyl-L-(4-iodo)phenylalanyl-(3*S*,4*S*)-statyl-L-leucyl-L-phenylalanylamide; statine is 4-amino-3-hydroxy-6-methylheptanoic acid; Boc, *N*-α-*tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; Sta, (3*S*,4*S*)-statine; MW, molecular weight; FW, formula weight; HBT, *N*-hydroxybenzotriazole hydrate.

Renin (EC 3.4.23.15) is an aspartic proteinase important to the control of blood pressure[1,2]. Renin inhibitors can lower blood pressure, but the reported compounds do not have all the attributes of clinically useful agents[3,4], such as oral activity and long duration of action. Structural studies of renin may be helpful in designing novel renin inhibitors as antihypertensive drugs. The renin isolated and purified from mouse

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submaxillary glands is the best characterized renin, with sequence[5,6] and some preliminary X-ray crystallographic work[7,8] reported. Mouse submaxillary renin is similar to human kidney renin, the renin of importance as a drug target. These two renins have about 70% sequence homology[9,10]. We[11] have isolated and purified mouse renin in large amount, and present herein spectroscopic and kinetic data to describe the stoichiometry, kinetics, and thermodynamics of the interaction of mouse renin with a potent renin inhibitor (**1**, see Figure 1) that is a substrate analog containing statine, a 3-hydroxylated gamma-amino acid which functions as a transition state or intermediate analog of the cleaved dipeptide unit.[12] The inhibitor potency of this compound and 15 analogs of it are given to illustrate the importance of various moieities of **1** for tight binding to renin. Since the binary complex of this inhibitor with mouse renin crystallizes in tetragonal bipyramids that diffract to better than 2.8 Angstroms[8], it is likely that the information presented here will be interpretable at the molecular level.

EXPERIMENTAL

Materials

Submaxillary glands from male Swiss-Webster mice were purchased from Pel-Freez. The major form of renin (renin A) was isolated and purified from the gland according to Poe *et al.*[11], a modification of earlier methods[13,14]. Synthetic tetradecapeptide renin substrate was from U.S. Biochemicals and fluorescamine from Sigma. Methanol and *N,N*-dimethylformamide were HPLC grade from MCB.

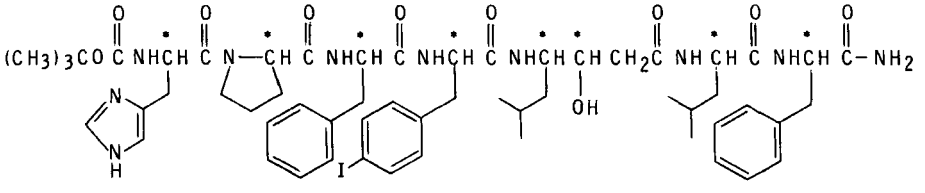
For the synthesis of **1**, Boc-Sta was prepared according to Rich *et al.*[15] Boc-(D,L)-*p*- Iodo-Phe was prepared from (D,L)-*p*-Iodo-Phe (Sapon Laboratories) using di-*tert*- butyldicarbonate and diisopropylethylamine in 2:1 tetrahydrofuran:water. Other Boc-amino acids were obtained from Chemalog or Bachem. Solvents and reagents were reagent grade and used without further purification, except for *N,N*-dimethylformamide, which was degassed under reduced pressure prior to use in syntheses.

Buffer A, 0.1 M citrate phosphate pH 7.2, was prepared by neutralizing 0.2 M Na_2HPO_4 with 0.2 M citric acid, followed by dilution to 0.1 M.

Synthesis

N-α-*tert*-butyloxycarbonyl-L-Histidinyl-L-Prolyl-L-Phenylalanyl-L-*p*-Iodophenylalanyl-(3*S*,4*S*)-4-amino-3-hydroxy-6-methyl-heptanoyl-L-Leucyl-L-Phenylalanyl Amide, **1** = Boc-His-Pro-Phe-*p*-I-Phe-Sta-Leu-Phe-NH_2, **1**.

The title peptide sequence was prepared by standard solid phase techniques[16] as a peptide resin ester, on a Beckman 990B Peptide Synthesizer. Beginning from a 2% cross-linked Merrifield resin esterified with



N-α-(t-BUTYLOXYCARBONYL)-L-HISTIDYL-L-PROLYL-L-PHENYLALANYL-
(4-IODO)-L-PHENYLALANYL-(3S,4S)-STATYL-L-LEUCYL-L-PHENYLALANYL AMIDE

$C_{57}H_{77}N_{10}O_{10}I$ F.W. 1189.21

FIGURE 1 Structure of compound **1**.

Boc-Phe (1.21 mmole/g, 1 mmole), subsequent Boc-amino acids were added sequentially, using 40% trifluoroacetic acid in CH_2Cl_2 for Boc-deprotection, 10% diisopropylethylamine in CH_2Cl_2 for neutralization, and 1 M DDC in CH_2Cl_2 for coupling. All couplings used 2.5 equivalents of Boc-amino acid, except as noted below, plus equimolar additive of HBT in a solvent of 1 : 1, CH_2Cl_2 : *N*,*N*-dimethylformamide. Couplings were for 30 min, followed by a recoupling cycle of 60 min. For couplings of Boc-Sta and Boc-(D,L)-*p*-I-Phe, 1.25 equivalents of the Boc-amino acid plus HBT were coupled for 6–18 h, followed by recoupling using the retained coupling solution, with no additional DCC added. This procedure preserved the supply of these amino acid derivatives while giving complete coupling. The N^{im}-dinitrophenyl protecting group on His was removed as the final resin step using 10% thiophenol in *N*,*N*-dimethylformamide.

The completed resin ester peptide was removed by aminolysis in 50 ml methanol saturated at 0°C, with ammonia, to which was added 1 g ammonium acetate. The suspension was stirred in a pressure bottle at room temperature for three days, effecting complete aminolysis. After release of the pressure, the resin suspension was washed repeatedly with methanol and CH_2Cl_2, and the filtrates were evaporated, redissolved in ethyl acetate, and washed with water. The organic layer was dried over Na_2SO_4, and evaporated to give 1.0 g of crude product, as a mixture of two diasteriomers, due to the (D,L)-*p*-I-Phe.

TLC on silica showed two spots of nearly equal intensity at R_f values of 0.68 and 0.64 in 80 : 20 : 2 : 1, chloroform : methanol : water : acetic acid. The diasteriomers were separated using 0.04–0.063 micron silica gel chromatography in 100 : 15 : 1.5 : 1 of the TLC solvent mixture. Pooling of pure fractions and precipitation from CH_2Cl_2/ether gave 0.228 g of pure upper TLC isomer (A), 0.275 g of pure lower TLC isomer (B) and 0.236 g of mixed isomers. Comparison of the ^{1}H-n.m.r. spectra at 360 MHz of A and B with those of analogs containing either D- or L-Phe in place of the *p*-I-Phe unambiguously identified isomer A as the L-*p*-I-Phe-containing diasteriomer.

Additional characterization of the L-*p*-I-Phe product: reverse phase HPLC, 94.3% pure; amino acid analysis, His 0.98, Pro 0.96, Leu 1.03, Phe 2.03, Sta and *p*-I-Phe not determined, 92% peptide based on MW = 1189.21; FAB MS, M + H at 1189 (single isotope MW = 1188), M − I + H at 1063; elemental analysis, $C_{57}H_{77}N_{10}O_{10}I$–$C_2H_4O_2$–$H_2O$, MW = 1189.21, FW = 1267.28, peptide content = 93.8%. (Found: C, 56.07; H, 6.63; N, 11.18; I, 10.32. Requires C, 55.92; H, 6.6; N, 11.05; I, 10.01%). The D-*p*-I-Phe diasteriomer, B, was characterized similarly. The D-*p*-I-Iodo-Phe analog was retained slightly longer than the L-compound on reverse phase HPLC.

The other fifteen compounds described in Table I were synthesized and characterized analogously to the above; details of their syntheses will be reported separately.

METHODS

Absorption and Difference Spectra

Absolute absorption spectra were measured on a Cary 118 spectrometer. Difference spectra were taken on the Cary 118 using the split compartment cell of Yankeelov[17] essentially according to earlier protocols[18,19]. Difference spectra were taken at ambient temperature (24°C.) on the 0.1 absorbance scale from 350 to 240 nm at a scan rate of 1 nm s^{-1}. The difference spectrum between **1** in methanol and **1** in buffer A was obtained by subtracting the absorbance of **1** in methanol versus methanol from the absorbance of **1** in buffer A versus buffer A.

Mouse renin concentrations were standardized by absorbance at 280 nm[13], using $\varepsilon(280) = 36\,700\ cm^{-1}\,M^{-1}$. Mouse renin, prepared as in Ref. 11 was 100% active; concentrations of mouse renin standardised by enzyme activity were equal to concentrations standardised by absorbance at 280 nm. The concentration of **1** was standardized by weight, using the formula weight of 1189.21 and assuming that the compound was 93.8% peptide by weight, a value derived from an elemental analysis of the hydrated acetic acid salt and confirmed by quantitative amino acid analysis and ^{1}H-n.m.r. on the compound. In methanol, the absorbance maximum of **1** was at 230 nm for $\varepsilon(230) = 20\,800\ cm^{-1}\,M^{-1}$ with a prominent shoulder at about 234 nm.

Fluorescence

Fluorimetric measurements were made on a Perkin–Elmer 650-10M fluorimeter at 25 ± 0.5°C. To ascertain the stoichiometry of binding, 2.00 ml of 1.6 μM mouse renin was placed in a 4-sided quartz cuvette and allowed to equilibrate to 25°C. This solution gave a strong fluorescence (about 100 units on the 0.3 scale) when observed at 350 nm (5 nm bandpass) with 290 nm excitation (5 nm bandpass). In the stoichiometric titration, five microliter aliquots of 100 μM **1** were serially added, and the fluorescence read after each addition.

In the measurement of the kinetics of binding of **1** to mouse renin, 2.00 ml of 1.6 μM mouse renin was placed in the sample cuvette and allowed to come to 25°C. Then, sixty microliters of 1.00 mM **1** in *N*,*N*-dimethylformamide was added to the enzyme solution, mixed rapidly and the fluorescence (excitation, 290 nm; emission, 350 nm) recorded versus time. A control experiment was done identically; except that sixty microliters of *N*,*N*-dimethylformamide were substituted for the solution of **1**. The binding kinetics were repeated at 0.25 μM mouse renin and 0.25 μM **1** (one microliter of 0.5 mM **1** in *N*,*N*-dimethylformamide).

Inhibition data

A fluorimetric renin assay[11] based on the methods of Corvol *et al.*[20] and Galen *et al.*[21], was used to measure the inhibition constant of **1** for cleavage of a synthetic tetradecapeptide renin substrate by mouse renin in buffer A at 37°C. The experimental data were expressed as relative activity, with 100% activity corresponding to the increase in fluorescence due to substrate cleavage seen in the absence of inhibitor. The experimental data was fitted to the equation $v = V/(S + K_M[1 + \{i/K_i\}])$ with v the observed fluorescence increase divided by the increase seen without inhibitor, V was v times $S + K_M$ when $i = 0$, S was substrate concentration (5.7 μM), K_M was the Michaelis constant (18.3 μM), i was the inhibitor concentration (0 to 5 nM for **1**) and K_i was the competitive inhibition constant.

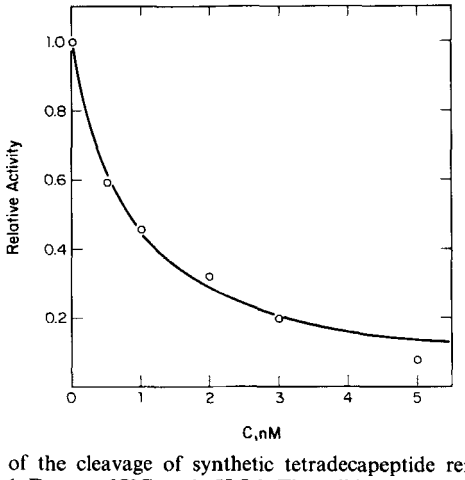


FIGURE 2 Inhibition of the cleavage of synthetic tetradecapeptide renin substrate by mouse submaxillary gland renin by **1**. Done at 37°C. and pH 7.2. The solid line is a theoretical curve for competitive inhibition with a K_i of 0.62 nM.

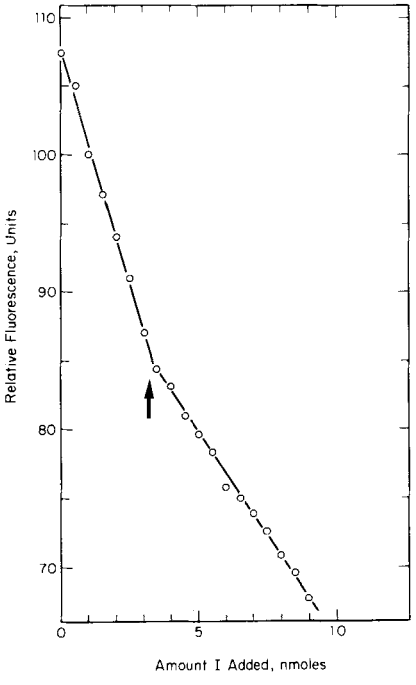


FIGURE 3 Quenching of tryptophan fluorescence of mouse renin by compound **1**. Titration done by adding aliquots of **1** in methanol to 3.2 nmoles of mouse renin at 25°C. and at pH 7.2. The arrow marks where one equivalent of **1** has been added.

RESULTS

Depicted in Figure 1 is the covalent structure of **1**. This heptapeptide with blocked amino- and carboxy-terminii contains two unusual amino acids, a γ-amino acid at position 5, (3*S*,4*S*)-3-hydroxy-4-amino-6-methylheptanoic acid, and a phenylalanine substituted with iodine at the para-position of the aromatic ring. All carbons marked with asterisks in Figure 1 are in the "S" or "L" configuration. The γ-amino acid, named statine by Umezawa *et al.*[22] was originally discovered in pepstatin, a potent inhibitor of aspartic proteases[23,24]. The absolute configuration of statine was determined by both X-ray analysis[25] and chemical synthesis.[26]

Incubation of mouse renin with 0.5–5 nM **1** showed a pronounced inhibition of the cleavage of synthetic tetradecapeptide renin substrate by the enzyme (see Figure 2). The open circles were data points in a typical titration. None of these data points differed significantly from the solid line, which was a theoretical curve for a competitive inhibitor with a K_i of 0.62 nM. The experimental data and the theoretical curve for a K_i of 0.62 nM also agreed within experimental error, when the experiment summarized in Figure 2 was repeated with four other substrate concentrations: 1.42, 2.85, 4.27, and 7.11 μM (data not shown). The D-*p*-Iodo-Phe-containing analog of **1** was determined to be at least a 900-fold less potent competitive inhibitor of mouse renin than **1**. Compound **1** was also a potent inhibitor of human renin, determined as described by Boger *et al.*[12], with a K_i of 3.5 nM.

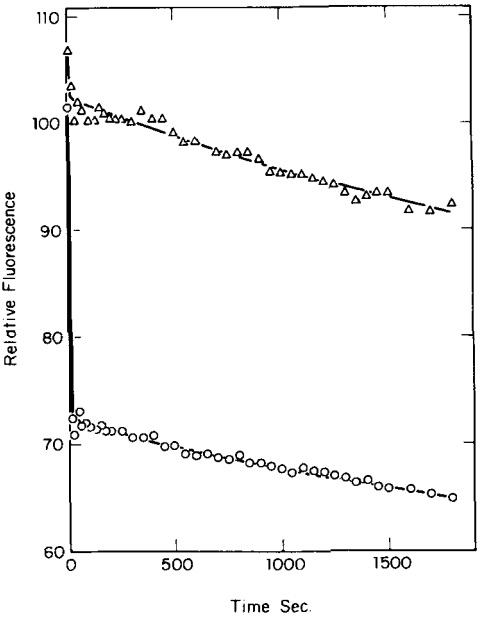


FIGURE 4 Time course of enzymic tryptophan fluorescence after addition of excess **1** in *N*,*N*-dimethylformamide (○) or of an equal volume of solvent (△). Done at 25°C. and pH 7.2.

To confirm the tight binding of **1** by mouse renin, a fluorecence titration was done (see Figure 3). Fluorescence was observed at 350 nm with excitation at 290 nm. Most of this fluorescence was probably due to the three tryptophan residues of mouse renin[5,6]. This fluorecence was partially quenched upon complexation of mouse renin with **1**. Due to the poor solubility of **1** in water at pH 7.2 (a saturated solution is about 30 μM at 25°C.), the compound was dissolved in methanol at 100 μM, and 5 μl aliquots were added serially to 2 ml of 1.6 μM renin (3.2 nmoles standardised by both enzyme activity and UV absorbance). Unfortunately, the solvent also slightly quenched the fluorescence of the enzyme, as is shown in the right-hand portion of Figure 3 for data above 3.2 nmoles of added **1**. It was clear that **1** was bound to mouse renin with a dissociation constant certainly below 20 nM and probably below 3 nM. The renin fluorescence decreased linearly with added **1** up to one equivalent of added **1**. This point of equivalence is indicated by the arrow in Figure 3. Further additions of **1** led to a linear decrease of fluorescence with a different slope. The decrease in fluorescence in this portion of the titration could be elicited with methanol addition alone. A dissociation constant of 20 nM would lead to easily-detectable curvature in the plot of fluorescence versus added **1** in Figure 3. A dissociation constant of 3 nM would probably have led to detectable curvature, but such curvature could have been masked by experimental error. It was also clear that mouse renin bound one mole of **1** per mole of renin. For a fuller discussion of the measurement of dissociation constants and binding stoichiometries from fluorescence titrations such as these, see Refs. 27 and 28.

One possible difficulty in the interpretation of kinetic and thermodynamic data for binding of **1** to renin would arise if **1** were very slowly bound by the enzyme. However, slow binding was not the case, as may be seen in the data replotted for

Figure 4. The data in circles corresponded to the addition of 60 μl of 1 mM **1** in *N*,*N*-dimethylformamide (29 μM final concentration of **1**) to 2 ml of 1.6 μM renin in buffer A, while the data in triangles corresponded to the addition of 60 μl solvent alone. The very rapid drop in fluorescence seen with complexation of **1** was complete within 15 s of addition. The slow phase of fluorescence decrease appeared to be due to solvent alone. Similarly, at 0.25 μM mouse renin and 0.25 μM **1**, the rapid drop in fluorescence was complete within 15 s of addition, followed by the slow, solvent-caused decreased which was linear with time for 10 min (data not shown). If the fluorescence values at times 350–600 s after addition of 0.25 μM were linearly extrapolated back to zero time, the three values measured within the first 15 s were on this extrapolated line, demonstrating that greater than 95% of the fluorescence decrease due to the binding of **1** occurred within 15 s. Since the dissociation constant for **1** from mouse renin was determined to be less than 3 nM in the fluorescence titration (and was probably near the inhibition constant K_i of 0.62 nM), an upper limit of 5 nM for the dissociation constant can be placed on the process being monitored in the rapid phase of the fluorescence kinetics.

The complexation of **1** by mouse renin led to an intense UV difference spectrum (see solid line in Figure 5). Replotted for Figure 5 is the difference in UV absorbance between the renin-**1** complex and its unmixed constituents. There are two principal

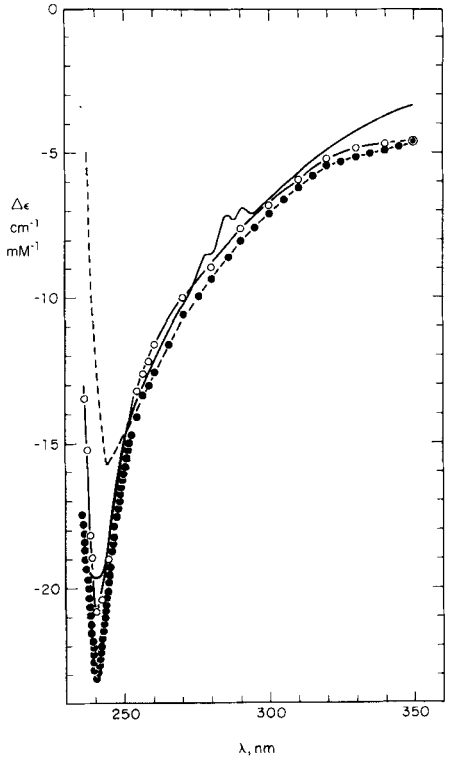


FIGURE 5 UV difference spectrum seen upon binding **1** to mouse renin (solid line). The solid line with circles (○) is the difference in absorbance between **1** in methanol and **1** in 0.1 M citrate phosphate buffer pH 7.2; the line in dashes is **1** in CH_2Cl_2 versus **1** in buffer and the dots are **1** in acetonitrile versus **1** in buffer.

features of this enzymic difference spectrum. First, there is a dramatic decrease in the broad, intense UV absorbance of the 4-iodophenylalanyl chromophore, with a change in extinction at 242 nm of $-19\,500\,cm^{-1}\,M^{-1}$. Second there is a triplet of enhanced absorption bands at 278, 285, and 291 nm, which corresponds to absorption maxima of tryptophan[29]. The $\Delta\varepsilon$ of the largest of these changes is about $+1100\,cm^{-1}\,M^{-1}$, after correction for the absorbance change due to the 4-iodophenyl chromophore. The solid line with circles (○) in Figure 5 corresponds to the UV absorbance of 5 μM **1** in methanol (versus methanol) minus the UV absorbance of 5 μM **1** in buffer A, adjusted mathematically to the same scale as the enzymic difference spectrum. Analogous plots for **1** in acetonitrile versus **1** in buffer A (data with dots) and for **1** in CH_2Cl_2 versus **1** in buffer A (data with dashes) are also shown in Figure 5. The similarity between the dashed UV difference spectrum in methanol and the enzymic difference spectrum shows that the binding of compound **1** by mouse renin possibly results from a transformation of the 4-iodophenyl chromophore of **1** from a water solution to a "methanol-like" environment.

DISCUSSION

There are several research groups making renin inhibitors (see, for example Refs. 12 and 30–35). As part of our renin inhibitor synthetic program[12,30,31], several compounds have been made to help solve the X-ray crystal structure of mouse submaxillary gland renin. The most useful of these compounds is **1**, whose structure is given in Figure 1. The compound is an analog of the octapeptide minimum renin substrate sequence, His-Pro-Phe-His-Leu-Leu-Val-Tyr. Statine replaces the scissile dipeptide, Leu-Leu, transforming the sequence into a transition-state analog[12,30,31]. Compound **1** is a highly potent competitive inhibitor of the cleavage of synthetic tetradecapeptide renin substrate by mouse submaxillary gland renin. It has a K_i of 0.62 nM; see Figure 2 and Table I. It is also a potent inhibitor of human kidney renin, with a K_i of 3.5 nM.

To understand better the contribution of the various moieities of **1** to its tight binding by mouse renin, 15 of the compounds made in our renin inhibitor synthetic program[12,30,31] were tested against mouse renin. The inhibitor potencies (K_i values) are given in Table I. The numbers 6–9 and 12–13 represent the analogous positions in the octapeptide minimum renin substrate sequence[31], and B (statine) represents the scissile dipeptide (positions 10 and 11 of the substrate sequence). The three different blocking groups used at A (Boc in **2**, Iva in **3**, and Ibu in **4**) were all about equal in their contribution to inhibitor potency. Substitution of an *o*-I-Phe at position 8, as in **5**, is tolerated by mouse renin with no change in binding. Substitutions at position 9 with aromatic residues (His in **2**, Phe in **6**, Tyr in **7**, and *p*-iodo-Phe in **1**) led to somewhat greater differences in binding to mouse renin, with compound **1** the most potent inhibitor of mouse renin yet reported. Deletion of residues at positions 13 (compound **8**), 12 and 13 (compound **9**) or at positions 6 and 7 (compound **10**) led to profound decreases in inhibitor potency. Replacement of leucine in position 12 with isoleucine (compound **11**) did not change inhibitor potency, but replacement with valine (compound **12**) or ornithine (compound **13**) reduced inhibitor potency by more than a factor of ten. Substitution of the phenylalanine in position 13 by tyrosine (compound **14**) or histidine (compound **16**) also decreased the inhibitor potency for mouse renin, but *p*-I-Phe (compound **15**) is nearly equivalent.

The last column in Table I summarizes the inhibitor potencies of the compounds

TABLE I
Structure-activity correlation for statine-containing, substrate-analog inhibitors of mouse submaxillary renin and human kidney renin[a]

No	Structure[b]									K_i, inhibition constant, nM	
	A	6	7	8	9	B	12	13	C	MSR	HKR
1	Boc	His	Pro	Phe	*p*IP	Sta	Leu	Phe	NH2	0.62	3.5
2	Boc	His	Pro	Phe	His	Sta	Leu	Phe	NH2	4.9	2.3
3	Iva	His	Pro	Phe	His	Sta	Leu	Phe	NH2	14	8.8
4	Ibu	His	Pro	Phe	His	Sta	Leu	Phe	NH2	15	7.7
5	Boc	His	Pro	*o*IP	His	Sta	Leu	Phe	NH2	3.4	23
6	Boc	His	Pro	Phe	Phe	Sta	Leu	Phe	NH2	5.0	0.78
7	Ibu	His	Pro	Phe	Tyr	Sta	Leu	Phe	NH2	3.8	5.3
8	Ibu	His	Pro	Phe	His	Sta	Leu	–	NH2	1700	170
9	Ibu	His	Pro	Phe	His	Sta	–	–	NH2	62 000	870
10	Boc	–	–	Phe	His	Sta	Leu	Phe	NH2	950	190
11	Iva	His	Pro	Phe	His	Sta	Ile	Phe	NH2	17	1.9
12	Ibu	His	Pro	Phe	His	Sta	Val	Phe	NH2	190	110
13	Iva	His	Pro	Phe	His	Sta	Orn	Phe	NH2	500	250
14	Boc	His	Pro	Phe	His	Sta	Leu	Tyr	NH2	100	4.4
15	Boc	His	Pro	Phe	His	Sta	Leu	*p*IP	NH2	11	2.9
16	Iva	His	Pro	Phe	His	Sta	Leu	His	NH2	270	28

[a]Inhibitors were dissolved in *N*,*N*-dimethylformamide and added as small aliquots (0.1–1%, v/v) to the enzyme solution. Enzyme assays were done in a volume of 1 ml at 37°C. in 0.1 M citrate phosphate pH 7.2 with 5700 nM synthetic tetradecapeptide renin substrate and for 16–24 h with 0.25 ng human kidney renin [HKR][45] or for 30–60 min with 200 ng mouse submaxillary gland renin [MSR][11].

[b]Abbreviations used in this table: Boc, *N*-α-tert-butyloxycarbonyl-; Iva, *N*-α-isovaleryl-; Ibu, *N*-α-Isobutyryl-; Sta, statyl or (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoyl-; *o*IP, *ortho*-Iodo-L-phenylalanyl-; *p*IP, *para*-Iodo-L-phenylalanyl-; Orn, L-ornithyl. Significant changes from compound **2** are underlined.

versus human kidney renin. While there are some interesting differences in inhibitor potency for compounds **1**, **5**, **7**, **14** and **16** for the mouse and human renins, it is clear that the two renins are similar in their binding sites for the octapeptide minimum renin substrate sequence, especially in binding substrate positions on the *N*-terminal side of the scissile bond. Thus, the crystal structure of the binary complex of **1** with mouse renin[8], when solved, should provide insight of direct relevance to human kidney renin.

The kinetic and thermodynamic studies we have made of the interaction of mouse renin with compound **1** show that compound **1** is bound rapidly and tightly, is bound one-to-one, and goes into a hydrophobic pocket.

The binding of **1** to mouse renin is quite rapid (see Figure 4). At either 29 μM **1** or 0.25 μM **1** binding is complete within 15 s. Assuming that the rate of binding of **1** by mouse renin is governed by the usual second order rate equation (see Ref. 36), first order with respect to two reactants of unitary stoichiometry, that is:

$$\text{renin} + \mathbf{1} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{renin-}\mathbf{1}$$

then:

$$-\mathrm{d[renin]}/\mathrm{d}t = -\mathrm{d[}\mathbf{1}\mathrm{]}/\mathrm{d}t = \mathrm{d[renin\text{-}}\mathbf{1}\mathrm{]}/\mathrm{d}t = k_{on}\,[\mathrm{renin}][\mathbf{1}] - k_{off}\,[\mathrm{renin\text{-}}\mathbf{1}] \quad (1)$$

with t = time; [renin], [**1**], and [renin-**1**] the concentrations of ligand-free renin, of free **1**, and renin-bound **1**, respectively; and with k_{on} and k_{off} the kinetic rate constants

for binding and dissociation, respectively. For [**1**] much larger than [renin], the concentration of [**1**] will not change much during binding and may be treated as a constant. For tight binding of **1** by renin, k_{off} is much smaller than k_{on} [**1**], so that the k_{off} [renin-**1**] term in Eq. (1) may be neglected. Therefore, to a good approximation, Eq. (1) may be solved as: [renin] = [renin at $t = 0$] $e^{-t/\tau}$, where τ, the exponential time constant for this pseudo first-order process, is equal to $1/(k_{on})$ [**1**]. The concentration of free renin drops to 0.4% of total renin in 5τ[36]. Therefore from the experimental results, $5\tau \leqslant 15$ sec, and k_{on} is therefore $\geqslant 1.3 \times 10^6\ s^{-1}\ M^{-1}$. This rapid binding is in marked contrast to the time-dependent inhibition seen by Rich *et al.*[37] for inhibition of pepsin, also an aspartic proteinase, by pepstatin and pepstatin analogs containing statine. The half-lives (1.45τ) for binding to pepsin of the pepstatin-like inhibitors with inhibition constants less than 10 nM are about 30 s. For pepsin, tightly-bound, pepstatin-like inhibitors are bound in a collision complex which rapidly converts to an intermediate complex with a dissociation constant K greater than 10 nM. This then converts slowly to a more-tightly bound complex. The molecular nature of the slow binding step to pepsin is unclear. For the binding of **1** by mouse renin, the rapidly-formed complex had a dissociation constant $\leqslant 5$ nM, and there was no time-dependent tightening seen.

The rapidity of binding of **1** to mouse renin allows the satisfactory fit of inhibition data for **1** to a competitive inhibition equation (see Figure 2). The fluorescence titration of mouse renin with **1**, depicted in Figure 3, confirms the tight binding shown in the inhibition data in Figure 2. The titration also shows that one mole of renin bound one mole of **1**. It is fortunate that the fluorescence shows the stoichiometry of binding so clearly, since the poor water solubility of **1** precludes use of many of the common techniques used for the assessment of stoichiometry.

Perhaps the most dramatic spectroscopic data obtained with **1** is the UV difference spectrum seen upon renin binding. It should be interesting to see what constitutes a "methanol-like" environment for the 4-iodophenyl moiety of **1**. The crystal structures of renin-related acid proteases from fungi, like rhizopuspepsin[38,39], penicillopepsin[40–42], and endothiapepsin[43], show a long deep hydrophobic cleft that appears to be the substrate and inhibitor binding site. The cleft may also be the fatty acid binding site in mouse renin[44]. This cleft is probably where **1** binds.

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