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Active Site Directed Inactivators of Mouse Submaxillary Renin†

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ABSTRACT: The following active site directed inactivators for the pressor enzyme renin were synthesized: L-α-bromoisocaproyl(BIC)-Leu-Val-Tyr-Ser-OH, L-BIC-Val-Tyr-Ser-OH, L-BIC-Leu-Val-OCH_3, L-BIC-Leu-Val-OH, L-BIC-Val-Tyr-NH_2, L-BIC-Val-Tyr-OCH_3, L-BIC-Val-Tyr-OH, L-BIC-Leu-OCH_3, L-BIC-Val-OCH_3, and L-BIC-OCH_3. The rate of inactivation of mouse submaxillary gland renin by these reagents was studied under a variety of conditions. L-α-Bromoisocaproyl-Val-Tyr-Ser-OH and L-α-bromoisocaproyl-Leu-Val-Tyr-Ser-OH were the most efficent inactivators followed by L-α-bromoisocaproyl-Val-Tyr-NH_2. The rates of inactivation by the first two peptides were strongly dependent on pH, being most efficient at low pH, least efficient at pH near 5.6, and becoming efficient again at netural pH. The rate of the inactivation by L-α-bromoisocaproyl-Val-Tyr-NH_2, in which the C-terminal carboxyl group is blocked, was only slightly dependent on pH. Complete inactivation was achieved by these three reagents. The inactivation was accompanied by incorporation of a stoichiometric quantity of the radiolabeled reagents. Based on these findings it was concluded that the inactivators reacted with a carboxyl group(s) in the active site of the renin molecule to form an esteric linkage. These data also suggest that a carboxyl group(s) may constitute part of the catalytically essential functional groups in renin action. D-α-Bromoisocaproyl derivatives of the various peptides mentioned above were also prepared. These compounds were much less active than the L isomers indicating that the inactivation by the L-α-bromoisocaproyl peptides was a specific reaction.

Renin (EC 3.4.4.15) plays a central role in the homeostatic control of blood pressure by producing angiotensin I through the limited proteolysis of the unique leucylleucine peptide bond of the plasma renin substrate. It also cleaves the same peptide bond in the tetradecapeptide renin substrate H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-*Leu-Leu*-Val-Tyr-Ser-OH (Skeggs et al., 1957). The mechanism of such a highly selective proteolysis is not understood. Experiments designed to characterize renin action using specific inactivators of proteases such as diisopropyl phosphorofluoridate (Pickens et al., 1965; Reinharz et al., 1971), *p*-hydroxymercuribenzoate (Reinharz et al., 1971), or EDTA (Pickens et al., 1965) have indicated that renin does not belong to a known class of protease such as the serine, cysteine, or metallo protease.

Systematic studies of Kokubu et al. (1968) of competitive inhibitors possessing part of the structure of the tetradecapeptide substrate (Skeggs et al., 1957) have indicated that the C-terminal portion Leu-Leu-Val-Tyr is the specific determinant for the binding to the active site of hog renal renin. Our preliminary studies support these findings. Furthermore, we have observed that the N-terminal portion of the tetradecapeptide such as angiotensin I has little affinity for renin.[1]

Although renins from different sources may be different to a certain extent, the extrarenal renin from the mouse submaxillary gland has been shown to have properties closely resembling renal renins (Cohen et al., 1972; Inagami et al., 1974). Furthermore, antibodies against the mouse submaxillary gland renin were found to cross-react with mouse renal renin (Michelakis et al., 1974). Thus, information obtained from studies of renal renin and submaxillary gland enzyme was interchangeably utilized in designing a series of active site directed inactivators possessing the general structure of α-bromoisocaproyl oligopeptide. The isocaproic acid possesses the hydrocarbon side chain identical with that of leucine, thus it can function as one of the leucine residues of the unique leucylleucine structure in directing the inactivators to the active site. These reagents were applied to the pure and stable preparation of renin from the mouse submaxillary gland (Cohen et al., 1972) for the purpose of characterizing its active site and as a method of specific inactivation of renin.

Materials and Methods

Materials. The peptides L-valyl-L-tyrosine, L-valyl-L-tyrosinamide, and L-leucyl-L-valine were obtained from Cyclo Chemical Co.; L-valyl-L-tyrosyl-L-serine and L-leucyl-L-valyl-L-tyrosyl-L-serine were purchased from Fox

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[1] Unpublished observation of Murakami and Inagami.

Table I: Bromoacylated Peptide Inhibitors of Mouse Submaxillary Renin. Yield of Synthesis, Melting and Boiling Points, and Elemental Analyses.

Compounds	Yield (%)	Mp(°C) (not cor)	Formula (mol wt)	Calcd (%)				Found (%)[a]			
				C	H	N	Br	C	H	N	Br
L-α-Bromoisocaproic acid	71	Bp 160–162 (6.7 mm)	$C_6H_{11}O_2Br$ (195.07)	36.94	5.69		40.97	37.09	5.80		40.75
L-BIC-L-Val-OH	45	125–127	$C_{11}H_{20}NO_3Br$ (294.2)	44.91	6.85	4.76	27.16	45.03	6.69	4.81	26.95
L-BIC-L-Val-L-Tyr-OH	43	158–160	$C_{20}H_{29}N_2O_5Br$ (457.1)	52.52	6.39	6.21	27.16	52.17	6.37	6.05	17.13
L-BIC-Val-Tyr-NH_2	71	203–204	$C_{20}H_{30}N_3O_4Br$ (456.4)	52.63	6.62	9.21	17.51	52.86	6.66	9.50	17.42
L-BIC-Val-Tyr-Ser-OH	61	136–138 dec	$C_{23}H_{33}N_3O_6Br\cdot{}^3/_2H_2O$ (554.5)	49.82	6.54	7.58	14.41	49.73	6.36	7.83	14.59
L-BIC-Leu-OH	44	125–126[b]	$C_{12}H_{22}NO_3Br$ (308.2)	46.78	7.20	4.54	25.93	46.94	7.43	4.36	26.03
L-BIC-Leu-Val-OH	12	185–186 dec	$C_{17}H_{31}N_2O_4Br\cdot{}^1/_2H_2O$ (407.4)	49.04	7.75	6.73		49.01	8.02	6.82	
L-BIC-Leu-Val-Tyr-Ser-OH	46	110–112	$C_{29}H_{45}N_4O_8Br$ (657.6)	52.97	6.90	8.52	12.15	52.96	6.71	8.30	11.91
D-α-Bromoisocaproic acid	65	Bp 113–115 (3.0 mm)	$C_6H_{11}O_2Br$ (195.07)	36.94	5.69		40.97	36.86	5.69		40.90
D-BIC-Val-Tyr-NH_2	56	205–207	$C_{20}H_{30}N_3O_4Br\cdot H_2O$ (474.4)	50.64	6.80	8.86	16.84	50.61	6.62	8.78	16.60
D-BIC-Val-Tyr-Ser-OH	38	135–136	$C_{23}H_{33}N_3O_6\cdot H_2O$ (545.4)	51.64	6.47	7.62		51.62	6.94	7.24	
D-BIC-Leu-Val-Tyr-Ser-OH	90	115–116	$C_{29}H_{45}N_4O_8Br$ (657.6)	52.97	6.90	8.52		52.85	6.84	8.39	
BP-Val-Tyr-NH_2	56	196–197	$C_{17}H_{24}N_3O_4Br$ (414.3)	49.28	5.84	10.14		49.39	5.64	10.14	
BP-Leu-Val-Tyr-Ser-OH	25	125–127	$C_{26}H_{39}N_4O_8Br$ (615.5)	50.72	6.39	9.10	12.98	50.79	6.29	8.94	12.76

[a] Analyses were performed by Galbraith Laboratories, Knoxville, Tenn. [b] Literature value, 128°, Fischer and Koelker (1907).

Chemical Co.; L-valine and L-leucine were products of Schwarz/Mann and D-leucine was obtained from Sigma Chemical Co. [4,5-^{3}H]L-Leucine was obtained from New England Nuclear Co. β-Bromopropionic acid was an Eastman product. The fluorogenic octapeptide substrate of renin (benzyloxycarbonyl-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-β-naphthylamide) of Roth and Reinharz (1966) was the gift of Dr. Roth. The mouse submaxillary renin fraction A was prepared by the published method (Cohen et al., 1972). Aminopeptidase M was purchased from Karl Roth Co., Karlsruhe, West Germany. Dioxane, 1-propanol, and ethylene glycol monomethyl ether were obtained from Fisher Scientific Co.

D- and L-α-Bromoisocaproic Acid. The α-bromo substituted isocaproic acids were prepared by a slight modification of the method of Fischer (1906). To an ice-cold suspension of L-(or D)-leucine (5.0 g) in 15 ml of cold 48% HBr (Fisher) was added with stirring 1 ml of Br_2. Following this NO gas was passed through the solution for 1 hr. After another addition of Br_2 (1 ml) the NO bubbling was continued for 1 more hr. The mixture was extracted with ethyl ether and the ether layer washed successively with 1% sulfuric acid, 10% sodium carbonate, and water and dried over anhydrous sodium sulfate overnight. After evaporation of the ether, the α-bromoisocaproic acid was obtained by distillation in vacuo as a colorless liquid with $[\alpha]^{20}D$ −6.10° (*c* 5.06, methanol) for L-α-bromoisocaproic acid and $[\alpha]^{20}D$ 6.19° (*c* 5.30, methanol) for the D enantiomer (see Table I for boiling points and elemental analyses). The acid chloride of α-bromoisocaproic acid was prepared by directly treating the acid (200 mg) with 2 ml of thionyl chloride. After allowing reaction for 2 hr at room temperature, thionyl chloride was evaporated under reduced pressure using a rotary evaporator. Traces of thionyl chloride were removed by reevaporation after addition of dry benzene. The tritiated derivatives of α-bromoisocaproic acid were prepared in a similar manner using [4,5-^{3}H]-L-leucine (New England Nuclear) and diluted appropriately with nonradioactive leucine. The purity of these peptides was checked by thin-layer chromatography on silica gel using two solvent systems: (1) 1-butanol–acetic acid–water, 18:2:5 (v/v); and (2) toluene–pyridine–water, 80:10:1 (v/v). All preparations gave a single spot.

α-Bromoisocaproyl (BIC[2]) Peptides. These peptides were prepared by dropwise addition of the acid chloride (1.5 mmol) of α-bromoisocaproic acid to ice-cooled solutions of peptides or amino acids (1.5 mmole in 2 ml). The addition took place over a period of 1.5 hr maintaining the pH between 10 and 11 with the use of 1 *N* NaOH. After an additional 30 min of reaction the pH was lowered to 2.5–3.0 with ice-cold 1 *N* HCl. The product was extracted from the solution with ethyl acetate. The ethyl acetate was evaporated under reduced pressure and the residues were recrystallized from methanol–petroleum ether. β-Bromopropionyl peptides were prepared similarly from β-bromopropionyl chloride and H-Val-Tyr-NH_2 or H-Leu-Val-Tyr-Ser-OH. The yields, melting points, and results of elemental analyses are summarized in Table I. Methyl esters of BIC peptides were prepared by esterifying parent BIC peptides in methanol–thionyl chloride. The radiolabeled peptides [4,5-^{3}H]BIC-Val-Tyr-NH_2 (599 cm/nmol) and [4,5-^{3}H]BIC-Leu-Val-Tyr-Ser-OH (1785 cpm/nmol) were prepared by these methods from [4,5-^{3}H]-L-leucine.

Reaction of Submaxillary Renin and Bromoisocaproyl Peptides. To 160 μl of 0.05 *M* sodium pyrophosphate buffer (pH 5.6) in a glass-stoppered centrifuge tube was added 20 μl of 5 m*M* solution of a BIC peptide in ethylene glycol monomethyl ether. After equilibration at 37° the reaction was started by the addition of 20 μl of 0.1% solution of mouse submaxillary renin A (Cohen et al., 1972) in the same buffer which also contained 0.1 *M* NaCl. For reactions at pH 4, 0.05 *M* sodium acetate instead of the pyrophosphate buffer was used.

Labeling of Renin with Tritiated BIC Peptides. A reac-

[2] Abbreviations used are: BIC, α-bromoisocaproyl; BP, β-bromopropionyl. The abbreviated designation of amino acid residues denotes the L form.

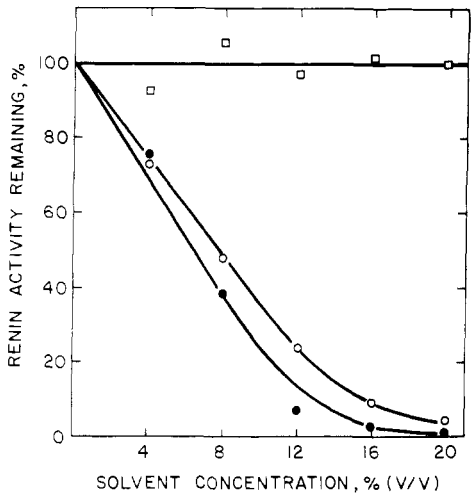

FIGURE 1: Effect of solvents on renin activity. Mouse submaxillary renin A was incubated in 0.04 *M* pyrophosphate buffer (pH 5.6) containing 1-propanol (●), dioxane (○) or ethylene glycol monomethyl ether (□) at 37° for 10 hr. Renin concentration was 0.108 mg/ml. Renin activity was determined by the method given in the text.

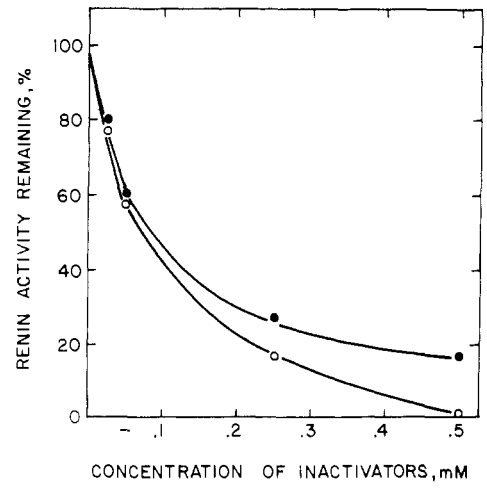

FIGURE 2: Effect of the concentration of BIC peptides on the inactivation of submaxillary renin. Mouse submaxillary renin A (0.1 mg in 1 ml) was incubated with L-BIC-Val-Tyr-NH_2 (○) or L-BIC-Leu-Val-Tyr-Ser-OH (●) in 0.04 *M* pyrophosphate buffer (pH 5.6) at 37° for 10 hr. The inactivators were added as solution in ethylene glycol monoethyl ether. The concentration of the organic solvent was 10% (v/v) throughout this experiment. Renin activity was determined by the method given in the text.

tion mixture consisting of 0.9 ml of renin fraction A (0.37 mg), 0.6 ml of a 5 m*M* solution of [^{3}H]BIC-Leu-Val-Tyr-Ser-OH or [^{3}H]-BIC-Val-Tyr-NH_2 in ethylene glycol monomethyl ether, and 4.5 ml of 50 m*M* acetate buffer (pH 4) or 5.0 m*M* pyrophosphate buffer (pH 7) was allowed to react at 37° for 24 hr followed by dialysis in the cold against 10 m*M* acetate buffer (pH 4.4) containing 10% ethylene glycol monomethyl ether. The solution was then concentrated by freeze-drying, dissolved in the 10 m*M* acetate buffer (pH 4.4), and passed through a column (2.5 × 42 cm) of Sephadex G-50 to remove free tritiated peptide.

Hydroxylamine Treatment of the Radiolabeled Renin. A solution (0.5 ml) containing the radiolabeled renin (200–2000 cpm) was treated for 24 hr at 25° and pH 9 with 2 ml of 1.25 *M* NH_2OH–10 *M* urea mixture which had been adjusted to pH 9.0 immediately before the reaction. The mixture was dialyzed exhaustively against 10 m*M* sodium acetate buffer (pH 4.0) containing 10% (v/v) ethylene glycol monomethyl ether, freeze-dried, and dissolved in 0.25 ml of the same buffer and the radioactivity was determined.

Determination of Renin Activity. The fluorogenic octapeptide substrate of Roth and Reinharz (1966) was used. The renin containing solution (10 μl) from the above reaction mixture was mixed with 230 μl of 0.05 *M* pyrophosphate buffer (pH 5.6) and the reaction was started by the addition of 10 μl of the substrate solution in dimethylformamide (0.5–1 mg/ml). The reaction was stopped after 1 hr by heating for 10 min in a boiling water bath. The procedure used to release β-naphthylamine from the substrate cleaved by renin was as follows. After cooling the reaction mixture (250 μl) to 37°, 10 μl of 0.1 *M* zinc acetate, 10 μl of 2 *M* Tris-HCl buffer (pH 8.6), and 100 μg (10 EU) of aminopeptidase M in 1% solution were mixed and the reaction was allowed to proceed for 2 hr at 37°. The concentration of the β-naphthylamine released was determined in a Farrand spectrofluorometer MK-1 using a 3 × 3 mm quartz cell according to Reinharz and Roth (1969).

Other Determinations. Radioactivity was determined using a Packard TriCarb scintillation counter. Samples (0.25 ml) were added to 10 ml of the scintillation fluid Aquasol (New England Nuclear). An internal standard consisting of labeled peptides was used to determine the degree of quenching. Protein concentrations were determined either spectrophotometrically ($E_{1\text{ cm, }280\text{ nm}}$(1%) 10.0) or by the method of Lowry (1951) using the standard renin solution as reference.

Results

Effect of Organic Solvent on Submaxillary Renin. Since the peptides used in these studies were not readily soluble in water, a solvent system for these peptides had to be found which would not have a deleterious effect on renin. The effects of dioxane, 1-propanol, and ethylene glycol monomethyl ether on renin activity were examined as the function of their concentration. As shown in Figure 1, ethylene glycol monomethyl ether (open squares) was found to have a negligible effect even at 20% (v/v) after 20 hr at 37°, whereas 1-propanol (filled circles) and *p*-dioxane (open circles) brought about a marked inactivation of the enzyme. Based on these observations, a 10% (v/v) mixture of ethylene glycol monomethyl ether in water was used as the medium for all of the peptides examined in the present studies.

Effect of the Concentration of Inactivator Peptides on the Rate of Inactivation. Experiments were carried out to determine the appropriate concentrations of these inactivators. The extent of inactivation by L-BIC-Leu-Val-Tyr-Ser-OH and L-BIC-Val-Tyr-NH_2 was determined after 10 hr of incubation at pH 5.6 and 37° as the function of the concentration of inactivator. The pH of 5.6 was used since renin activity as determined by the fluorometric assay is highest at this pH. Results shown in Figure 2 indicate that 0.5 m*M* L-BIC-Val-Tyr-NH_2 can completely inactivate the mouse submaxillary renin in 10 hr. On the other hand, the longer peptide L-BIC-Leu-Val-Tyr-Ser-OH is less effective. However, as shown in Figure 3, it also can inactivate renin completely after a longer incubation time at a different pH. Based on these observations the inactivation of renin by the remaining peptide derivatives was examined at a concentration of 0.5 m*M*.

The time course of the inactivation of the submaxillary renin A by L-BIC-Leu-Val-Tyr-Ser-OH was studied at different pH values. As shown in Figure 3 the inactivation was

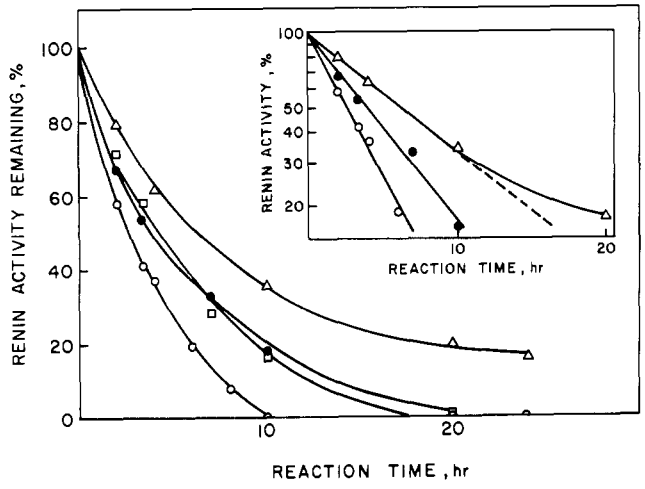

FIGURE 3: Time course of the inactivation of submaxillary renin by L-BIC-Leu-Val-Tyr-Ser-OH at different pH. Inactivation reactions were carried out in a manner similar to Figure 2 at 37° and at a 0.5 m*M* inactivator concentration. Aliquots of reaction mixtures were assayed directly using the fluorogenic octapeptide substrate of Roth and Reinharz (1966) as described in the text. The inset shows semilogarithmic plots of the same results. Buffers (0.04 *M*) used were acetate at pH 4 (○), pyrophosphate, pH 5 (●) and pH 6 (△), and phosphate at pH 7 (□). At pH 4 and 7 additional 0.04 *M* pyrophosphate was added for the stabilization of renin.

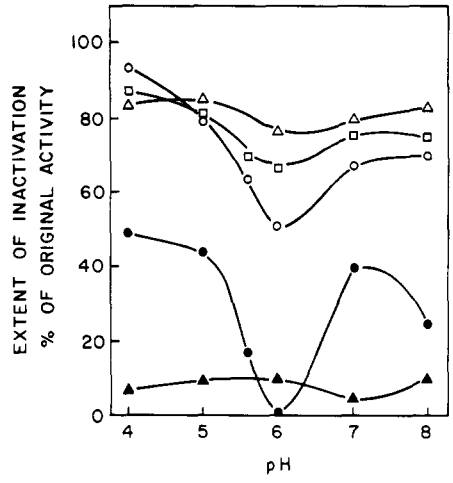

FIGURE 4: Extent of inactivation of submaxillary renin by different L- and D-bromoisocaproyl peptides at different pH. Inactivators used are: L-BIC-Leu-Val-Try-Ser-OH (○), L-BIC-Val-Tyr-Ser-OH (□), L-BIC-Val-Tyr-NH_2 (△), D-BIC-Leu-Val-Tyr-Ser-OH (●), and D-BIC-Val-Tyr-NH_2 (▲). Reaction conditions and buffers are identical with those given for Figure 3. Pyrophosphate buffer (40 m*M*) was used for pH 8. Inactivators were added as solutions in ethylene glycol monomethyl ether to a final concentration of 0.5 m*M* and the final ethylene glycol monomethyl ether concentration was 10% (v/v). After 10.5-hr reactions at 37° and given pH, the inactivators were dialyzed out immediately by the same buffer containing the 10% solvent in the cold. The activity was assayed by the method given in the text.

practically complete in 10 hr at pH 4 under the conditions employed. As the inset shows, the inactivation follows pseudo-first-order kinetics during the first 10 hr. Thus it was possible to compute the first-order rate constants for various peptides as will be discussed later. The rate of the inactivation was highest at pH 4, lowest at pH 6, and increasing again at pH 7–8. Since this inactivator L-BIC-Leu-Val-Tyr-Ser-OH has an ionizing carboxyl group, it was probable that the varying ionic interaction due to the ionization could have resulted in such a biphasic pH dependence.

The pH dependence of five additional inactivators was also examined in these studies. The activity remaining after 10.5 hr of reaction with the various inhibitors was determined over a pH range from 4 to 8. Plots of the extent of inactivation against pH (Figure 4) indicate that the inactivation by the peptide with a free C-terminal carboxyl group was indeed dependent on pH, being highest at pH 4, lowest at a pH of approximately 6, and again higher at neutral pH. The inhibitory activity of L-BIC-Leu-Val-Tyr-Ser-OH (open circles) was somewhat more strongly dependent on pH than that of L-BIC-Val-Tyr-Ser-OH (open squares). On the other hand, the rate of inactivation by a peptide not containing a free carboxyl group (L-BIC-Val-Tyr-NH_2) (open triangles) was almost independent of pH. In general BIC derivatives of valyltyrosyl peptides were more effective than the leucylvalyltyrosyl derivative. D-BIC derivatives had some inhibitory activity although at considerably reduced levels as compared with their L counterparts. The pH dependence of the reactivity of the D-BIC peptides was quite analogous to that observed with their L counterparts. The inactivation by D-BIC-Leu-Val-Tyr-Ser-OH (filled circles), though much less efficient than the L-BIC peptide, was strongly dependent on pH, whereas the action of the least efficient inactivator D-BIC-Val-Tyr-NH_2 (filled triangles) was practically independent of the pH. This was further confirmed by reactions carried out for 20 hr.

The observations noted above suggested that the inactivating ability was not limited to one specific structure. These investigations were extended to compounds of various related structures and the first-order rate constants of the inactivation of renin by these compounds were determined at pH 4 and 5.6. The pH 4 is the lowest pH value attainable without danger of denaturing the submaxillary renin during the prolonged incubation. It is also the pH at which the inactivation by certain compounds is most efficient. At pH 5.6 renin is most active in our assay conditions with the octapeptide substrate. Results shown in Table II indicate that longer peptides are generally more efficient than shorter peptide derivatives. Although D-BIC peptides inactivate renin to a certain extent, the reactivity of the L-BIC peptides is much more pronounced most probably because they react in a stereospecific manner. However, it is puzzling that the inhibitory activities of L-BIC-Leu-Val-Tyr-Ser-OH and L-BIC-Val-Tyr-Ser-OH do not differ appreciably either at pH 4 or 5.6. It may be that the two peptides are reacting with two different functional groups of renin. The alternative explanation may be that neither of the peptides directs the bromoisocaproyl group to the position most favorable for its reaction with the carboxyl group in the active site. L-BIC-Leu-Val-OCH_3 has a somewhat higher rate constant than L-BIC-Leu-Val-Tyr-Ser-OH or L-BIC-Val-Tyr-Ser-OH in spite of the fact that the first peptide has no tyrosyl residue. Masking of free C-terminal carboxyl group considerably increases the rate of inactivation as can be seen from the comparison of L-BIC-Leu-Val-OCH_3 with L-BIC-Leu-Val-OH and also from the comparison of L-BIC-Val-Tyr-NH_2 with L-BIC-Val-Tyr-OH. The D-BIC peptides have a considerably reduced rate of inactivation, particularly D-BIC-Val-Tyr-NH_2. This peptide is practically devoid of inactivating activity. The peptides D-BIC-Leu-Val-Tyr-Ser-OH and D-BIC-Val-Tyr-Ser-OH are somewhat more reactive. Even methyl L-α-bromoisocaproate (L-BIC-OCH_3) has a considerable effect. But L-α-bromoisocaproic acid and bromoacetic acid with a free carboxy

Table II: Inactivation of Mouse Submaxillary Renin A by α-Bromoisocaproyl Peptides.[a]

Inhibitors	Pseudo-First-Order Rate Constant $k \times 10^2$ (hr^{-1}) pH 5.6	pH 4.0
L-BIC-Leu-Val-Tyr-Ser-OH	8.3	28
D-BIC-Leu-Val-Tyr-Ser-OH	1.5	5.2
L-BIC-Val-Tyr-Ser-OH	8.2	25
D-BIC-Val-Tyr-Ser-OH	3.2	4.9
L-BIC-Leu-Val-OCH_3	11	
L-BIC-Leu-Val-OH	3.7	
L-BIC-Val-Tyr-NH_2	20	22
D-BIC-Val-Tyr-NH_2	0.9	1.0
L-BIC-Val-Tyr-OCH_3	7.6	
L-BIC-Val-Tyr-OH	4.4	
L-BIC-Leu-OCH_3	9.5	9.7
L-BIC-Val-OCH_3	6.3	
L-BIC-OCH_3	6.1	6.0
D-BIC-OCH_3	1.0	
L-BIC-OH	0.5	
D-BIC-OH	0.5	
α-Bromoacetic acid	0.5	

[a] Reaction mixtures containing 40 m*M* pyrophosphate buffer (pH 5.6) or 40 m*M* acetate buffer (pH 4.0), 500 μ*M* inactivator, 3 μ*M* enzyme, and 10% (v/v) ethylene glycol monomethyl ether were incubated at 37° for 10.5 hr. After dialysis against the pyrophosphate buffer containing 10% (v/v) ethylene glycol monomethyl ether, the renin activity was determined by the method given in the text.

group have very little activity. Noteworthy is the fact that β-bromopropionyl peptides listed at the bottom of Table II reacted with submaxillary renin though at considerably reduced rates.

Evidence for the Initial Reversible Binding of BIC Peptides to the Active Site. A proof for the binding of the BIC peptides to the active site of the submaxillary renin was obtained from the study of the reversible inhibition of the renin by the BIC peptide in short term experiments. When various BIC peptides were added to a 0.5-ml reaction mixture containing 1.8 μg of submaxillary renin A, 0.04 m*M* octapeptide substrate, 10% (v/v) ethylene glycol monomethyl ether, and 40 m*M* sodium pyrophosphate buffer (pH 5.6), a considerable reduction in the reaction rate was observed in short term experiments lasting for 30 min at 37°. L-BIC-Val-Tyr-NH_2 added to a final concentration of 0.5 m*M* caused a 78% inhibition; D-BIC-Val-Tyr-NH_2 (0.5 m*M*), 32% inhibition; and L-BIC-Val-Tyr-Ser (10 m*M*), 52% inhibition. Since the irreversible inactivation by the BIC peptides under similar conditions, as estimated from data in Table II and Figure 3, should be less than 10% of the total enzyme activity, the extensive reduction in renin activity observed in the short term experiments lasting only for 30 min should be largely due to the reversible inhibition by the BIC peptides. As a more concrete proof for the largely reversible nature of the inhibition observed in these short term experiments, the recovery of renin activity upon dilution of the renin–BIC peptide mixture was investigated. After a 30 min incubation of renin at 37° in the above mentioned mixture containing a BIC peptide but not the octapeptide substrate, the solution was diluted with 10 volumes of the same buffer containing the octapeptide at 0.04 m*M* and the extent of the hydrolysis of the substrate was determined after 5 hr of reaction at 37°. Comparison with controls similarly treated but without BIC peptides showed that the renin treated with L-BIC-Val-Tyr-NH_2 recovered 77% of its activity upon dilution, D-BIC-Val-Tyr-NH_2, 93%, and L-BIC-Val-Tyr-Ser, 82%. The extensive restoration of the renin activity upon the 20-fold dilution indicates that a large part of the inhibition observed in the 30-min reaction was indeed due to reversible inhibition of submaxillary renin by the BIC peptides. These experiments show that BIC peptides are initially bound to the active site of renin in a reversible manner which is then followed by irreversible inactivation.

Stoichiometry of the Inactivators. A number of different functional groups in a protein can react with α-bromocarbonyl compounds (see, for example, Gurd, 1967). In order to show that the inactivation observed above was due to specific reaction between the inactivator and the enzyme, stoichiometric studies of the incorporation of the reagent to renin were carried out. The [4,5-^{3}H]L-BIC derivatives of H-Val-Tyr-NH_2 and H-Leu-Val-Tyr-Ser-OH were used for this purpose. After reaction for 24 hr under the conditions given in Table II, unreacted labeled peptides were removed by exhaustive dialysis followed by gel filtration on a column of Sephadex G-50. The incorporation of the peptides was practically stoichiometric. L-BIC-Val-Tyr-NH_2 is capable of inactivating renin A completely in less than 12 hr as can be seen from the result presented in Figure 1. However, the data given in Table III indicate that the prolonged reaction for 24 hr, twice as long as that required for the complete inactivation, did not cause a greater than stoichiometric incorporation of the reagent.

Characterization of the Functional Group Modified by the Inactivators. The α-halo carbonyl compounds can alkylate amino, imadazole, thioether, and sulfhydryl groups of proteins (Gurd, 1967). They can also esterify an especially reactive carboxyl group (Takahashi et al., 1967). The esters can be distinguished from other products since they can be dissociated by a hydroxylamine treatment.

The submaxillary renin A which has been completely inactivated by the stoichiometric incorporation of the radioactive peptide was treated with 1 *M* hydroxylamine in the presence of 8 *M* urea at 25° and pH 9 under the conditions employed by Takahashi et al. (1967). After 24 hr

Table III: Stoichiometry of the Incorporation of Inactivator into Mouse Submaxillary Gland Renin A.

Inhibitor	pH of Reaction	Specific Radioactivity (cpm/μmol) Inactivated Renin	Inactivator[a]	Molar Ratio Inhibitor/ Protein	Loss of Enzyme Activity (%)
[^{3}H]-L-BIC-Val-Tyr-NH_2	4	606,300	599,700	1.01	100
[^{3}H]-L-BIC-Leu-Val-Tyr-Ser-OH	4	1,705,000	1,785,000	0.99	100
[^{3}H]-L-BIC-Leu-Val-Try-Ser-OH	7	1,944,000	1,785,000	1.09	100

[a] Specific radioactivity of free inactivators. See the text for methods.

practically all radioactivity had been released from protein as indicated in Table IV. Treatment at pH 9 and 8 *M* urea but without hydroxylamine or treatment with 1 *M* hydroxylamine alone without urea resulted in an incomplete removal of radioactivity.

Inactivation by BP Peptides. Inactivation of mouse submaxillary renin by BP-Val-Tyr-NH_2 and BP-Leu-Val-Tyr-Ser-OH was studied at pH 5.6 under conditions identical with those employed for BIC peptides. Renin was inactivated by approximately 40 or 50% within the first few hours of the incubation. The pseudo-first-order rate constant of the initial phase could be estimated as 7.2×10^{-2} hr^{-1} for BP-Val-Tyr-NH_2 which was in the range comparable to those of BIC peptides. However, prolonged reaction did not cause further inactivation. After 10 hr approximately 45% of activity remained. After 20 hr no further change was observed.

Table IV: Hydroxylamine Treatment of Submaxillary Gland Renin A Inactivated with Tritium-Labeled Peptides.[a]

Inactivator Peptide Used for Labeling	Radioactivity Bound to Enzyme Protein Counts per 10 min	
	Before Hydroxylamine Treatment	After Hydroxylamine Treatment
[^{3}H]-L-BIC-Val-Try-NH_2	2,345	186
[^{3}H]-L-BIC-Leu-Val-Tyr-Ser-OH	17,149	108

[a] Renin A, 1.2 nmol, labeled with the [4,5-[^{3}H]-L-BIC peptides by the method given for Table III, was treated with 1 *M* hydroxylamine at pH 9.0 and 25° in a 2.5-ml reaction mixture containing 8 *M* urea for 24 hr. Details are given in text.

Discussion

Although renin is a peptidase, its highly restricted substrate specificity and its lack of susceptibility to a number of known types of protease inhibitors had suggested that this enzyme might be a peptidase of a special isolated type. Present studies were initiated in order to obtain some insight in this totally uncharacterized yet physiologically very important enzyme. The report of Kokubu et al. (1968) that the C-terminal tetra- or pentapeptide structure H-Leu-Val-Tyr-Ser-OH or H-Leu-Leu-Val-tyr-Ser-OH of the tetradecapeptide renin substrate (H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-OH) can inhibit renin action in vivo to a significant extent has suggested the possibility that such peptides may be incorporated in the synthesis of a specific active site directed inactivator of renin. This idea was further strengthened by our preliminary observation that these peptides also inhibited mouse submaxillary renin in vitro in a competitive manner.[1] On the other hand, the N-terminal octapeptide (angiotensin II) and decapeptide (angiotensin I) of the tetradecapeptide has only negligible inhibitory activity or binding affinity for the submaxillary renin.[1] α-Halocarboxylic acids can react with a variety of functional groups (Gurd, 1967; Takahashi et al., 1967). Although the reactivity of these reagents is not very great, they seemed to be suitable reagents for the initial phase of chemical modification studies attempting to characterize the active site of this enzyme. The α-bromoisocaproyl (BIC) group was chosen as the reactive function since it has a structure closely resembling that of leucine. Thus, in the design of the inactivators, BIC was employed to replace one of the two leucyl residues essential for a renin substrate.

Inactivators thus prepared consisted mostly of hydrophobic amino acid residues and presented solubility problems in their application to the inactivation experiment. Although most organic solvents had a irreversible deleterious effect on the mouse submaxillary renin, ethylene glycol monomethyl ether was found not to have any harmful effect on this enzyme at a concentration of 20% (v/v) during a prolonged incubation. Repeated experiments with peroxide-free or unpurified solvents confirmed these findings. Thus ethylene glycol monomethyl ether has been very useful in the present studies. It will be interesting to see if it will be equally useful in studies using a renal renin.

Results presented in Figure 3 demonstrate the L-BIC peptides synthesized in these studies are capable of inactivating mouse submaxillary renin completely. The time course of the inactivation follows the pseudo-first-order kinetics for approximately 10 hr. The deviation after 10 hr may be due to hydrolytic loss of inactivators during prolonged incubation. The specificity of the inactivation reaction is demonstrable by several findings. First, D-BIC-Val-Tyr-NH_2 has little or negligible inactivating capability compared with L-BIC-Val-Tyr-NH_2 (Table II). Second, L-BIC-OH or bromoacetic acid does not inactivate the renin (Table II). Third, the reaction is stoichiometric and prolonged reactions do not result in a greater than stoichiometric incorporation of the radiolabeled inactivator (Table III). Furthermore, the reversible inhibition of renin activity by several BIC peptides observed in short term experiments indicates that the BIC peptides are indeed bound to the active site of the enzyme.

On the other hand, there are observations which suggest a certain lack of specificity of other inactivators. The efficiency of inactivation by L-BIC-Leu-Val-Tyr-Ser-OH and L-BIC-Val-Tyr-Ser-OH is almost identical both at pH 4 and 5.6. The valine and tyrosine residues have been reported to make significant contributions to the substrate binding (Skeggs et al., 1968, Kokubu et al., 1968). If the valine and tyrosine residues of these BIC derivatives are bound at their respective binding sites of the renin, then the reactive α-bromocarbonyl group of the two peptides should be located at quite different positions, and yet these peptides have almost identical reactivity. Furthermore, degree of reactivity of the L-BIC-Leu-OCH_3, L-BIC-Val-OCH_3, and L-BIC-OCH_3 are not much different from each other at pH 5.6. Also it must be noted that corresponding D-BIC peptides, D-BIC-Leu-Val-Try-Ser-OH and particularly D-BIC-Val-Tyr-Ser-OH, can also inactivate this enzyme to a certain extent. These findings may be explained if it is assumed that the α-bromocarbonyl group of these two peptides may not occupy the most favorable position for the inactivation reaction.

Studies on the pH dependence of the rate of inactivation shown in Figure 4 indicate there are two groups of inactivators, one with little dependence on pH and the other having a biphasic dependence. The pH "independent" reagents are L- and D-Val-Tyr-NH_2, L-BIC-Leu-OCH_3, and L-BIC-OCH_3 all of which contain blocked C-terminal carboxyl groups. However, since the site modified by the reagents is a carboxyl group, the negligible dependence of the rate of inactivation on pH is puzzling. On the other hand, all the pH dependent reagents examined have free carboxyl groups at the C-terminal. It is most likely that the C-terminal carboxylate group somehow impedes the binding of the peptide to the active site region and that only at an acidic pH,

where it loses the negative electrical charge, does the reagent become efficient. The increase in the reactivity of these reagents at neutral pH is not easy to explain. Although one may postulate that the carboxyl group of the active site which is to be modified by the reagent is more reactive in its ionized carboxylate form than its neutral carboxyl form, such a hypothesis is not compatible with the pH-independent reactivity of BIC-Val-Try-NH_2. Alternatively the biphasic pH profile may be due to reaction involving two carboxyl groups in the active site of renin as in the case of pepsin (Fry et al., 1968; Bayliss et al., 1969; Fruton, 1971; Hartsuck and Tang, 1972). The pH profile of renin catalysis was found to be influenced profoundly by the size of the substrate[1] indicating that the reactivity and ionization of the catalytically essential functional groups are greatly influenced by the interaction of the enzyme with different types of substrates or inactivators. This variability may be the cause of the puzzling pH profile of inactivation reaction observed in the present studies.

The hydroxylamine treatment at pH 9 of the inactivated renin releases the radiolabeled inactivator peptide almost completely. This reaction is highly specific for the cleavage of an esteric linkage. Thus the present result clearly indicates that the group modified by the BIC derivative was a carboxyl group and the inactivation was due to the esterification of this carboxyl group by the displacement of the bromine atom of the BIC peptides. Preliminary evidence for the involvement of a particularly reactive carboxyl group has been obtained in our laboratory (Inagami et al., 1974) using diazoacetyl-D,L-norleucine methyl ester (Rajagopalan et al., 1966). Thus the involvement of at least one carboxyl group in the active site of renin is now clearly demonstrated. Whether the carboxyl group modified by the L-BIC peptides and the carboxyl group modified by the aliphatic diazo compound were identical or different would be an interesting problem to be elucidated in future studies.

BP-Val-Tyr-NH_2 and BP-Leu-Val-Tyr-Ser-OH were able to partially inactivate submaxillary renin. Although the inactivation was partial and the renin activity never decreased below 40% of the native level, it was unexpected that the β-bromopropionyl derivative reacted with the enzyme protein. However, the fact that complete inactivation was not obtained suggests that the reaction involving the β-bromopropionyl peptides took place with a functional group different from that which reacted with α-bromopeptides.

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