Characterization of the Active Site of Mouse Submaxillary Gland Renin†

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ABSTRACT: Renin plays a central role in blood pressure regulation. Due to the lack of a pure preparation, the nature of this enzyme has long been obscure. Mouse submaxillary gland has been shown to be closely analogous to renal renin and can be prepared in larger quantity than the renal enzyme. Catalytically essential groups of mouse submaxillary gland renin were identified by group-specific chemical modifications. Renin was completely inactivated by the acid protease specific inactivators, diazoacetyl amino acid methyl ester derivatives of glycine, norleucine, and leucine in the presence of the cupric ion, and by 1,2-epoxy-3-(*p*-nitrophenoxy)propane with a concomitant stoichiometric incorporation of the reagents. The incorporated reagent moiety in either of the above inactivation reactions could be removed by hydroxylamine treatment, indicating the esteric nature of the linkages. These two types of inhibition reactions were not mutually exclusive. These observations indicate that renin, analogous to acid proteases, carries two different catalytically essential carboxyl residues in its active site, each of which can be selectively modified by the two different types of the inactivators. Tyrosine-specific reagents tetranitromethane, acetylimidazole, and diazonium 1*H*-tetrazole inactivated renin completely. Pepstatin exerted strong protection against the inactivation with concomitant preservation of two tyrosyl residues, suggesting that at least two tyrosyl residues are located in the active center and may possibly by involved in the enzyme activity. Modification of arginyl residues by phenylglyoxal also resulted in complete inactivation of renin. Pepstatin protected one arginyl residue, suggesting that this residue is located near the active center and is required for the enzyme activity. Modification of histidyl, tryptophanyl, and amino groups had no effect on renin activity. Reactions with iodoacetic acid and *N*-ethylmaleimide revealed that renin has a partially buried free sulfhydryl group which is not essential for its enzyme activity. These results indicate that renin shares certain catalytic features with acid protease and, at the same time, has its own unique features.

Renin is a peptidase whose only known function is to catalyze the formation of the decapeptide angiotensin I by limited proteolysis of the single unique Leu–Leu peptide bond in its prohormone angiotensinogen, thus playing a key role in blood pressure regulation. Its unique role in the specific conversion of angiotensinogen to angiotensin I and its highly restricted substrate specificity have aroused interest of investigators concerning the nature of its catalytic mechanism and active site.

Attempts made in the past to characterize renin by the application of type-specific protease inactivators to crude enzyme preparations suggested that this enzyme does not share features with serine, cysteine, and metalloproteases (Haas et al., 1963; Pickens et al., 1965; Reinharz et al., 1971; Rubin, 1972). Inhibition by the acid protease inhibitor pepstatin (Aoyagi et al., 1972; Gross et al., 1972; Miller et al., 1972; Corvol et al., 1973; McKown et al., 1974; Overturf et al., 1974) suggested the possibility that renin is an acid protease. However, Aoyagi et al. (1972) reported that inhibition of acid proteases and renin occurs by different mechanisms mediated by two different residues in the pepstatin molecule. Moreover, the neutral pH optimum of renin is in sharp contrast to that of acid proteases. The highly stringent substrate specificity is not a property shared by acid proteases. Thus, the nature of renin has remained largely unknown. This is because of the lack of a pure preparation. Although pure renal renins have been prepared recently (Murakami & Inagami, 1975; Inagami & Murakami, 1977; Corval et al., 1977; Galen et al., 1979; Matoba et al., 1978; Slater et al., 1978; Yokosawa et al., 1978; Dzau et al., 1979), these were not available in quantities sufficient for the characterization of the active site. Mouse submaxillary gland renin can be purified in a relatively large quantity. This enzyme is closely related to mouse renal renin in several specific features (Michelakis et al., 1974). We used this renin as the model of renal renin to delineate the detailed features of the active site of renin.

Materials and Methods

Enzyme. Mouse submaxillary gland renin A was prepared by the method of Cohen et al. (1972). Renin activity was determined by the method of Reinharz & Roth (1969) using synthetic octapeptide renin substrate obtained from Bachem. The enzyme concentration was determined by optical absorbance at 280 nm with an $E_{1cm}^{1\%}$ = 10.0, by amino acid analysis, and by the method of Lowry et al. (1951) using pure renin as the standard.

Modification Reagents. Diazoacetyl amino acid methyl esters were prepared by the method of Bayliss et al. (1969). N_2Ac-Nle-OCH_3[1] was prepared from Gly-DL-Nle (Bachem) through its methyl ester hydrochloride and was recrystallized from methanol–ether (mp 64–47 °C); N_2Ac-Leu-OCH_3 was prepared as an oil from Gly-L-Leu (Bachem); [*acetyl*-1-^{14}C]N_2Ac-Gly-OCH_3 (mp 128.5–129 °C; 66.1 μCi/mol) was prepared from [*glycyl*-1-^{14}C]Gly-Gly (Schwarz/Mann). EPNP and phenylmethanesulfonyl fluoride were purchased from Eastman, and diisopropyl phosphorofluoridate and *N*-ethylmaleimide were from Sigma Chemical Co. Iodoacetic acid (Sigma) was recrystallized from hexanes before use. Pepstatin was obtained from Peninsula Laboratories. *N*-[*acetyl*-1-^{14}C]Acetylimidazole was prepared by the method of Reddy et al. (1963) and recrystallized from benzene (mp 103.5–104 °C; sp radioact 53 cpm/nmol).

Modification Reaction. Reaction of renin with the aliphatic diazo compounds N_2Ac-Nle-OCH_3, N_2Ac-Leu-OCH_3 and N_2Ac-Gly-OCH_3 was carried out according to the method of

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[1] Abbreviations used: N_2Ac, diazoacetyl; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; TNM, tetranitromethane; DHT, diazonium 1*H*-tetrazole; HNBS$(CH_3)_2$, dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium chloride.

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Rajagopalan et al. (1966). Details are given in the legend to Figure 1.

Reaction with EPNP (5 mg of powder per mL) was carried out by the method of Tang (1971) with renin solutions (3.2 mg/mL) in 0.2 M sodium acetate buffer, pH 4.8, at 4, 14, and 25 °C with gentle rotatory mixing. Reaction in the presence of pepstatin was preceded by incubation of renin with saturated pepstatin solution (68 μg/mL) at 23 °C and pH 4.8 for 16 h.

Reaction with iodoacetic acid (0.1 M) was performed in the dark with 1.17 mg/mL renin in 0.05 M sodium acetate buffer, pH 5.6, at 23 °C for 3 h. Enzyme activity was assayed after exhaustive dialysis against 0.05 M ammonium acetate buffer. *S*-(Carboxymethyl)cysteine was determined by amino acid analysis. Reaction with *N*-ethylmaleimide (20 mM) was done with 1 mg of renin in 1 mL of 0.1 M sodium phosphate buffer, pH 6.9, at 23 °C for 3 h by the method of Riordan & Vallee (1967). *S*-(Ethylsuccinimido)cysteine was determined by the method of Guidotti & Konigsberg (1964).

Nitration with TNM was performed according to Sokolowsky et al. (1966). Details are given in Figure 2. Acetylation with *N*-acetylimidazole was done by the method of Riordan & Vallee (1963) at pH 7.5 and 23 °C for 1 h. Acetylated renin was freed from the reagent by gel filtration through a column of Sephadex G-25. An aliquot of acetylated renin was treated with 0.1 M hydroxylamine at pH 6.7 for 15 h and dialyzed exhaustively against 0.01 M sodium phosphate buffer, pH 6.7. Deacetylation of *O*-acetyltyrosine by hydroxylamine, as monitored by an increase in absorbance at 278 nm, was completed in ~10 h. The number of *O*-acetyl groups was estimated by ^{14}C radioactivity released by hydroxylamine and by the spectrophotometric method of Simpson et al. (1963). The content of *N*-acetyl groups was calculated from the radioactivity remaining after the hydroxylamine treatment. Reaction with freshly prepared DHT was performed with 100–1000-fold excesses of DHT at pH 8.8 and 23 °C for 30 min according to the method of Takenaka et al. (1969).

Modification of arginyl residues by phenylglyoxal was carried out essentially by the method of Takahasi (1968). (See Figure 4 for details.) Ethoxyformylation of histidyl residues was done by the method of Melchior & Fahrney (1970). Tryptophanyl residues were modified with $HNBS(CH_3)_2Cl$ (Calbiochem) by the method of Horton & Tucker (1970). Amidination of amino groups was done by using ethyl acetimidate hydrochloride (Aldrich) by the method of Hunter & Ludwig (1962). Amino acid analyses were performed with 0.5–1-mg duplicate samples hydrolyzed in 6 N HCl at 110 °C for 24 h.

Results

Inactivation of Renin by Diazoacetyl Amino Acid Methyl Esters. The specific inactivator of acid proteases, N_2Ac-Nle-OCH_3 (Rajagopalan et al., 1966), added to an 80-fold molar excess to renin with cupric acetate (40-fold excess) completely inactivated renin in 2 h at pH 5.4 and 14 °C (open squares, Figure 1 inset). Cu(II) was essential for the inactivation as indicated by open circles.

With the objective of obtaining a renin-specific inhibitor, three different diazoacetyl amino acid derivatives, N_2Ac-Nle-OCH_3, N_2Ac-Leu-OCH_3, and N_2Ac-Gly-OCH_3, were compared for their inactivating ability. N_2Ac-Gly-OCH_3 was the most reactive reagent (filled circles, Figure 1 inset). N_2Ac-Leu-OCH_3, though least reactive, was able to inactivate renin completely in 4 h (filled triangles).

The extent of renin inactivation by these diazoacetyl derivatives was linearly related to the amount of the reagent

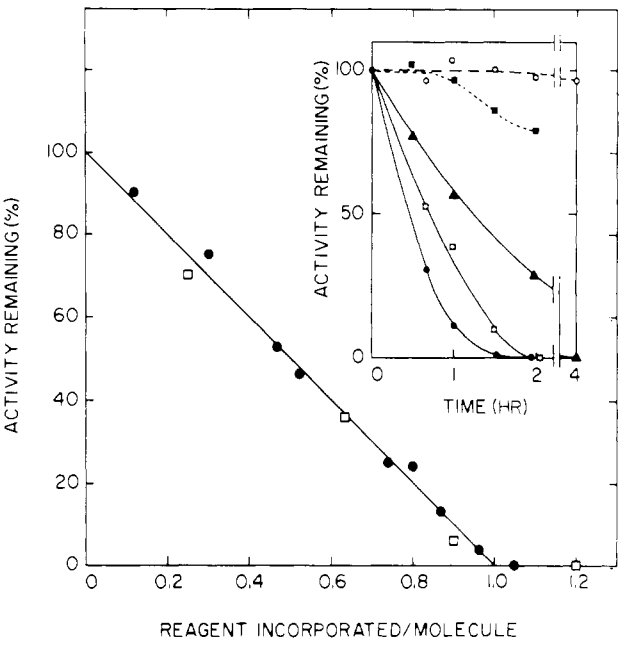

FIGURE 1: Relationship of change in renin activity and the extent of modification of renin by the diazoacetyl amino acid methyl esters N_2Ac-Nle-OCH_3 and [*acetyl*-1-^{14}C]N_2Ac-Gly-OCH_3. Renin (0.9 mg/mL) and an 80-fold molar excess of the diazoacetyl derivative at 2 mM were placed in 0.02 M sodium acetate buffer, pH 5.4. After preincubation for 5 min at 14 °C, a 40-fold molar excess of cupric acetate (1 mM) was added to start the reaction. Aliquots were withdrawn at intervals. The reaction was stopped by adding a 10-fold molar excess of EDTA over cupric ion. Modified renin was dialyzed against 0.05 M ammonium acetate buffer, pH 5.6, for 18 h and assayed for renin activity. Incorporated norleucine was determined by amino acid analysis (□), and the N_2Ac-Gly-OCH_3 derivative was determined by ^{14}C radioactivity (●). Inset: inactivation of renin by diazoacetyl amino acid methyl esters in the presence and absence of Cu(II) and pepstatin. Inactivation reactions were carried out in the same manner as described above using N_2Ac-Nle-OCH_3 (□), N_2Ac-Gly-OCH_3 (●), and N_2Ac-Leu-OCH_3 (▲). Control experiments were performed with N_2Ac-Nle-OCH_3 in the absence of cupric acetate (○). The reaction with N_2Ac-Nle-OCH_3 in the presence of pepstatin (■) was carried out in the same manner except that a hypersaturating concentration of pepstatin (68.5 μg/mL) was added to renin 15 min prior to the reaction.

incorporated into the enzyme protein, and the incorporation of 1 mol of the reagent per mol of renin was sufficient for the complete inactivation as shown in Figure 1.

Treatment of renin completely inactivated by either N_2Ac-Nle-OCH_3 or [*acetyl*-1-^{14}C]N_2Ac-Gly-OCH_3 with 0.2 M hydroxylamine at pH 7.0 and 23 °C for 24 h, followed by dialysis, reduced protein-bound norleucine and ^{14}C radioactivity to less than 0.5 and 1% of the untreated control, respectively, indicating the esteric nature of the linkages between the reagents and renin.

Inclusion of pepstatin in the reaction mixture of renin with N_2Ac-Nle-OCH_3 resulted in considerable protection of the enzyme. Approximately 80% of the activity remained after the reaction for 2 h (filled squares, Figure 1 inset) whereas complete inactivation occurred in this period in the absence of pepstatin (open squares).

Inactivation of Renin by EPNP. The epoxide inhibitor of pepsin EPNP (Tang, 1971) was also found to inactivate renin. A complete inactivation occurred in 192 h at 25 °C. At 14 °C a few percent of renin activity still remained after 216 h, while at 4 °C more than 60% of the activity remained after the same period.

At 25 °C, inactivation of renin was accompanied by the incorporation of essentially two EPNP residues per molecule. However, at 14 °C, incorporation of approximately one residue

Table I: Stoichiometry of the Reaction of EPNP with Resin

reaction				
temp (°C)	time (h)	inactn (%)	EPNP incorpd (mol/mol of renin)	molar ratio of incorpd EPNP to inactive renin
25	72	82	1.78	2.2
	120	98	1.88	1.9
14	144	95	0.931	1.1
	216	98	1.23	1.3
4	48	12	0.164	1.4
	96	28	0.256	0.91
	144	29	0.338	1.2
	216	39	0.418	1.1

Table II: Effect of Pepstatin on Inactivation of Renin by EPNP

reaction		without pepstatin		with pepstatin	
temp (°C)	time (h)	EPNP inactn (%)	EPNP incorpd (mol/mol)	EPNP inactn (%)	EPNP incorpd (mol/mol)
25	120	98	1.9	44	1.5
14	216	98	1.2	33	0.63
4	216	39	0.42	10	0.13

of EPNP per renin molecule resulted in almost complete inactivation (Table I). Likewise, at 4 °C, a practically one-to-one stoichiometric relationship was observed between the extent of inactivation and the extent of incorporation of EPNP (as shown in the last column of Table I) though complete inactivation was not reached in the reaction period examined. Apparently, only one residue, essential for renin activity, reacts with EPNP at lower temperatures whereas an additional residue becomes reactive at 25 °C, a situation quite analogous to that encountered in pepsin by Tang (1971) and Hartsuck & Tang (1972).

Treatment of EPNP–renin containing 0.93 EPNP residue per molecule (obtained by reaction at 14 °C for 144 h) with 0.05 M hydroxylamine for 96 h in 0.05 M sodium pyrophosphate buffer at pH 8.0 and 23 °C reduced the amount of protein-bound EPNP residue to 0.07 per molecule. The result suggests the esteric nature of the linkage between EPNP and renin.

Pepstatin was found to protect renin from inactivation by EPNP (Table II). In the presence of pepstatin, only 33% inactivation was obtained after reaction at 14 °C for 216 h whereas 98% inactivation occurred in its absence. Similar protection was also observed at both 4 and 25 °C. Furthermore, pepstatin prevents the inactivation and the modification to the same extent as can be seen by comparing the difference between the third and fifth columns with the difference between the fourth and sixth columns in Table II. Pepstatin seems to protect the catalytically essential carboxyl group, which is reactive at lower temperature, but not the second residue which becomes reactive at 25 °C.

In order to determine whether diazoacetyl derivatives and the epoxide react with the same or different carboxyl residues of renin, we carried out two series of consecutive modification experiments. In reaction series I (Table III), renin which had incorporated 1.13 residues of the diazoacetyl reagent [*acetyl*-1-^{14}C]N_2Ac-Gly-OCH_3 after reaction lasting for 3 h at pH 5.4 was subjected to a second-stage reaction with EPNP for 120 h at 25 °C and pH 4.8. The doubly treated renin incorporated 1.81 EPNP residues per molecule. In the inverse sequency of reaction (reaction series II), the first treatment of renin with EPNP under the same conditions resulted in an

Table III: Stoichiometry of Sequential Reactions with EPNP and [*acetyl*-1-^{14}C]N_2 Ac-Gly-OCH_3

	first reagent	no. of reagents incorpd per molecule	second reagent	no. of reagents incorpd per molecule
reaction series I	[*acetyl*-1-^{14}C]-N_2 Ac-Gly-OCH_3	1.13	EPNP[a]	1.81
reaction series II	EPNP[a]	1.88	[*acetyl*-1-^{14}C]-N_2 Ac-Gly-OCH_3	1.23

[a] The reaction with EPNP was carried out at pH 4.8 and 25 °C for 120 h. The reaction with [*acetyl*-1-^{14}C]N_2 Ac-Gly-OCH_3 was carried out in 0.05 M sodium acetate buffer (pH 5.4) at 14 °C and the enzyme concentration of 1.0 mg/mL, 2 mM [*acetyl*-1-^{14}C]N_2-Ac-Gly-OCH_3, and 1 mM cupric acetate for 3 h.

incorporation of 1.88 EPNP residues. The second reaction with [*acetyl*-1-^{14}C]N_2Ac-Gly-OCH_3 led to the incorporation of 1.23 residues of the radioactive reagent per molecule. Thus, the extents of reaction of N_2Ac-Gly-OCH_3 before (1.13 residues per mol of enzyme) and after (1.23 residues per mol) the modification of the other carboxyl group by EPNP are practically identical. Likewise, modification by EPNP before (1.88 residues per mol of enzyme) and after (1.81 residues per mol) the reaction with the diazoacetyl compound proceeds to the same extent. These results indicated that each of these two reagents reacts with different residues independently and that these reactions are not mutually exclusive.

Sulfhydryl Groups. The sulfhydryl reagents sodium iodoacetate (0.1 M) allowed to react at pH 5.6 and 23 °C for 3 h in the dark and *N*-ethylmaleimide (2 mM) at pH 6.9 and 23 °C for 3 h failed to affect renin activity. Amino acid analysis of renin treated with iodoacetic acid revealed that 0.99 residue of carboxymethyl group was incorporated into cysteine per renin molecule as (carboxymethyl)cysteine. Renin reacted with 0.02 M *N*-ethylmaleimide, however, contained only 0.22 residue of *S*-succinylcysteine. Since the conditions employed for the reaction with iodoacetic acid and *N*-ethylmaleimide were such that all the exposed sulfhydryl groups will be completely modified (Riordan & Vallee, 1967), the difference in the number of the incorporated reagents is most likely due to the steric interaction of the reagent molecules with the environment of the sulfhydryl group. The results, therefore, suggest that one free sulfhydryl group exists in a renin molecule in a partially buried state.

Nitration of Tyrosyl Residues. Nitration with TNM resulted in complete inactivation of renin. Figure 2 shows the time course of inactivation and concomitant nitration of tyrosyl residues in the reaction with a 120-fold molar excess of TNM. Greater than 95% of the activity was lost in 2 h with concomitant nitration of four tyrosyl residues.

The content of 3-nitrotyrosine determined spectrophotometrically agreed well with the value obtained by amino acid analysis. Furthermore, the sum of the 3-nitrotyrosine and tyrosine content approximated the 16 residues per molecule previously determined in the native submaxillary gland renin, indicating no appreciable side reactions. 3,5-Dinitrotyrosine was not detected in the amino acid analysis. Spectrophotometric titration at 428 nm of inactivated renin containing approximately four nitrotyrosyl residues per molecule gave a pK_a of 6.75 with an isosbestic point at 381 nm, demonstrating the characteristic ionization behavior of 3-nitrotyrosyl residues

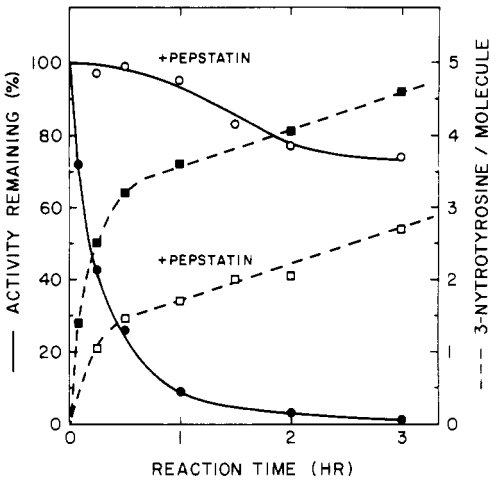

FIGURE 2: Nitration reaction with tetranitromethane in the absence and in the presence of pepstatin. A 120-fold molar excess of TNM was added as an ethanol solution to the renin solution (1 mg/mL) in 0.05 M Tris-HCl buffer, pH 8.3. Aliquots were withdrawn at intervals, and the reaction was terminated by shifting the pH to 5.0 by the addition of a precalibrated volume of 1 N HCl. After dialysis against 0.05 M sodium pyrophosphate buffer, pH 8.5, the number of 3-nitrotyrosine residues per protein molecule (■) and renin activity (●) were determined. The nitration reaction in the presence of 2.7 mM pepstatin was carried out after preincubation of renin with pepstatin for 15 min. Nitrated renin (in 0.5-mL aliquots) was freed from the reagents and pepstatin by passing through a Sephadex G-25 column (0.9 × 2.5 cm) in 0.05 M Tris-HCl buffer; 3-nitrotyrosine (□) and renin activity (○) were determined as described above.

(Sokolovsky et al., 1966). Gel filtration analysis of the above sample on a column of Sephadex G-100 (1.5 × 95 cm) gave a single protein peak with an apparent molecular weight of 38 000, suggesting that no significant intermolecular cross-linking was produced by TNM.

The nitration reaction proceeded in a biphasic fashion, and the initial rapid nitration of three to four tyrosyl residues was associated with loss of enzymatic activity (Figure 2). Further nitration at a slower rate was observed in the later period. The reaction with a 240-fold molar excess of TNM for 3 h resulted in nitration of eight to nine tyrosyl residues per molecule (filled squares, Figure 3a). However, as shown in Figure 3a, the degree of inactivation was linearly related to the nitration of the first four tyrosyl residues.

Pepstatin added to the reaction mixture resulted in a considerable protection of renin activity as shown in Figure 2. In the presence of pepstatin, reaction with a 120-fold molar excess of TNM for 3 h resulted in only 20–25% inactivation in contrast to the complete inactivation obtained in its absence. The course of the nitration reaction was again biphasic, and the slower phase of the reaction was parallel with the corresponding phase observed in the absence of pepstatin. Extrapolation of the slower portions of these two reaction curves gave a difference of 1.85 nitrotyrosine residues per molecule, suggesting protection of two of the four fast-reacting tyrosyl residues in the renin molecule.

Acetylation with Acetylimidazole. The reaction of renin with 100–800-fold molar excesses of [*acetyl*-1-^{14}C]acetylimidazole at pH 7.5 was completed in 1 h as monitored by changes in the renin activity. The extent of inactivation was a function of the amount of acetylimidazole initially added to the reaction mixture. An 800-fold molar excess of acetylimidazole was required for complete inactivation. The number of incorporated *O*-acetyl groups estimated from the hydroxylamine-labile ^{14}C radioactivity generally agreed well with the results obtained by the spectrophotometric method. As shown in Figure 3b, the loss of enzyme activity was again linearly related to the extent of the modification of tyrosyl residues and also to that of amino groups. Approximately four tyrosyl residues and seven amino groups were acetylated in completely inactivated renin.

Treatment of renin completely inactivated by acetylation with 0.1 M hydroxylamine at pH 6.7 and 23 °C for 15 h resulted in restoration of 70–80% of the original enzyme activity.

Diazo Coupling Reaction with DHT. The reaction with DHT (100–1000-fold molar excess) was completed in 30 min as monitored by changes in activity. The extent of inactivation was dependent on the amount of DHT. The inactivation was again linearly related to the modification of tyrosyl residues up to four residues per molecule (Figure 3c), in close agree-

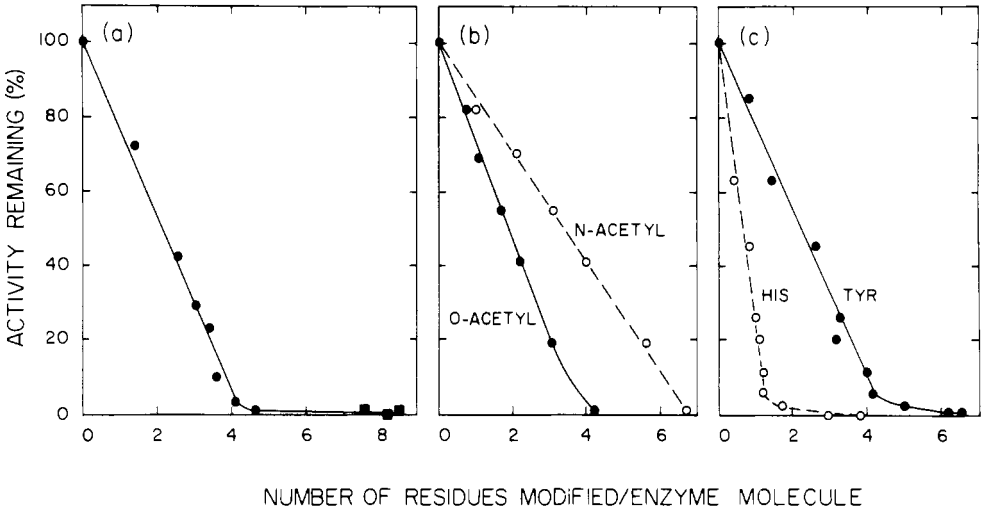

FIGURE 3: (a) Relationship of renin activity to the extent of nitration of tyrosyl residues by tetranitromethane. The nitration reaction was performed with a 120-fold excess (●) and a 240-fold excess (■) of TNM as in Figure 1. (b) The effect of O- and N-acetylation on renin activity by [*acetyl*-1-^{14}C]acetylimidazole (100–800-fold excess). (c) Modification of tyrosyl and histidyl residues by diazonium 1*H*-tetrazole and its effect on renin activity. The reaction was carried out at 23 °C and at a renin concentration of 0.7 mg/mL in 0.35 M borate buffer, pH 8.8, with varying amounts of diazonium 1*H*-tetrazole (100–1000-fold excess). The pH was maintained at 8.8 by the addition of 3 N NaOH delivered from a pH stat. After reaction for 30 min, sodium azide was added (40 mM) to stop the reaction. The sample was dialyzed against 0.05 M Tris-HCl buffer, pH 8.0, and adjusted to a fixed volume. Aliquots were used for renin assay, and numbers of modified tyrosyl and histidyl residues were estimated spectrophotometrically.

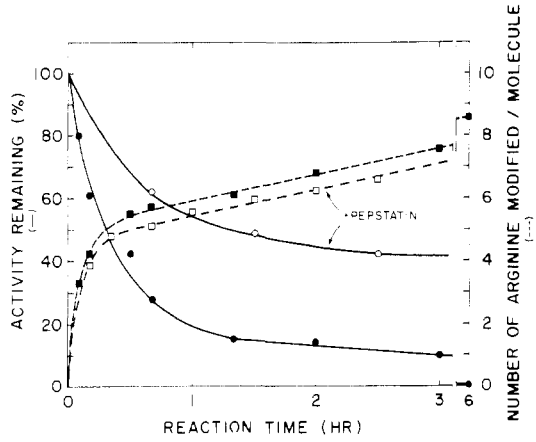

FIGURE 4: Time course of the modification reaction of renin with phenylglyoxal. Renin (1.3 mg/mL) was treated with 0.3% phenylglyoxal in 0.2 M *N*-ethylmorpholine buffer at pH 8.5 and 23 °C. Addition of phenylglyoxal was made with an aliquot of the stock solution (30%) made in 2-methoxyethanol. A portion of aliquots withdrawn at intervals was used directly for the assay of renin activity (●), and another portion was immediately acidified to pH 3.6 by adding a predetermined volume of glacial acetic acid, dialyzed against 0.01 N HCl, lyophilized, and subjected to amino acid analysis after hydrolysis in 6 N HCl for the determination of the extent of the modification of arginine residues (■). The reaction in the presence of a saturating concentration of pepstatin was carried out after preincubation of renin with pepstatin for 10 min. Modified renin (in 0.5-mL aliquots) was freed from the reagents and pepstatin by gel filtration on a Sephadex G-25 column (0.9 × 25 cm) in 0.05 M Tris-HCl buffer (pH 8.0). (○) Renin activity remaining; (□) the number of modified arginyl residues in the presence of pepstatin.

ment with the result obtained with TNM and acetylimidazole. Approximately 1.2 residues of histidine were modified concomitantly.

Modification of Arginyl Residues with Phenylglyoxal. Figure 4 shows time courses of inactivation and modification of arginyl residues in the reaction carried out with 0.3% phenylglyoxal. Inactivation was accompanied by progressive modification of arginyl residues. Complete inactivation was attained in 6 h with concomitant modification of 8.6 arginyl residues per molecule. At a higher concentration of the reagent (1.5%), complete inactivation was attained in 2 h.

Pepstatin exerted a significant protective effect; 40% of the original activity remained after 3 h of reaction as compared with 10% in its absence (Figure 4). Furthermore, a persistent difference of 0.5–0.7 residue in the number of modified arginyl residues per molecule was observed in the presence and absence of pepstatin throughout the parallel course of reaction, indicating that this peptide protects one arginyl residue of renin which apparently is essential for the enzyme activity. There was no detectable change in the compositions of other amino acids.

Ethoxyformylation of Histidyl Residues. Renin which had been treated with the histidine modification reagent ethoxyformic anhydride (400-fold molar excess) exhibited a characteristic difference spectrum of (ethoxyformyl)histidine against native renin used as reference with a positive maximum at 238 nm (Mühlrad et al., 1969). From the difference spectrum, the number of ethoxyformylated histidyl residues was estimated to be 7.8 residues per molecule. Ethoxyformylated renin retained ~92% of its original activity, indicating that histidyl residues are not involved in renin catalysis. It was confirmed by separate spectrophotometric experiments that the *N*-ethoxyformyl group on histidine residues of the enzyme molecule remained stable during incubation for 30 min under the conditions used for renin activity assay which lasted for 10 min.

Modification of Tryptophanyl Residues. Reaction with the tryptophan-specific reagent $HNBS(CH_3)_2Cl$ (300-fold molar excess) resulted in modification of 0.7 out of 2 tryptophanyl residues present in the renin molecule but had no effect on renin activity, indicating that tryptophanyl residues are only partially accessible and are not involved in renin catalysis.

Amidination of Amino Groups. Treatment of renin with an amino group (both α and ϵ) specific reagent, ethyl acetimidate (10000-fold excess), had no significant effect on renin activity. Although the extent of amidination was not determined, the reaction conditions were such that all the exposed amino groups were completely amidinated (Hunter & Ludwig, 1962; Nureddin & Inagami, 1975). The result, therefore, indicates that amino groups are not essential for the enzyme activity.

Serine protease specific inactivators, diisopropyl phosphorofluoridate (10 mM) and phenylmethanesulfonyl fluoride (1 mM) (incubated with renin in 0.05 M sodium phosphate buffer, pH 7.5, for 18 h), had no significant effect on renin activity. Similarly, dialysis of renin against 0.1 M EDTA and assay in the presence of 4 mM EDTA showed no alteration of the enzyme activity.

Discussion

The present studies demonstrate that at least two carboxyl groups, two tyrosyl residues, and one arginyl residue are essential for the enzymatic function of renin or are located in its active site. These conclusions are supported by the complete inactivation of the enzyme by specific modification of these residues and protection of the enzyme from the inactivation by the specific inhibitor pepstatin with a stoichiometric sparing of the residues in question from the modification.

The two carboxyl groups possess characteristics unique to highly reactive, catalytically essential functional groups. These features include elevated and specific reactivity with the diazoacetyl compounds and epoxides, the one-to-one stoichiometric relationship between the extent of chemical modification and the extent of inactivation by the diazoacetyl compounds and epoxides at low temperatures, the absolute requirement of cupric ion for the reaction with the diazoacetyl compounds, and complete release of the incorporated reagents by hydroxylamine for both types of modification. It is remarkable that modifications of these two residues are not mutually exclusive.

Practically identical features have been observed in the modification of the acid proteases pepsin (Rajagopalan et al., 1966; Tang, 1971), penicillopepsin (Sodek & Hofmann, 1968), and chymosin (Takahashi & Chang, 1973) by these acid protease specific reagents. Cupric ion dependent inactivation by aliphatic diazo compounds seems to be the common feature of acid proteases (Delpierre & Fruton, 1965). Thus, present results strongly indicate that renin has the two active-site carboxyl groups similar to those of acid proteases.

Modification of the EPNP-reactive carboxyl group proceeds at practically identical speeds independent of prior modification of the diazo compound reactive carboxyl group as shown by the results in Table II. Likewise, reaction of the latter with N_2Ac-Gly-OCH_3 is not affected by previous modification using EPNP. These results indicate independent reaction of two carboxyl groups. Similar observations have been reported in the study of pepsin by Tang (1971). The EPNP-specific carboxyl group of pepsin was shown to be different from the carboxyl group specifically modified by N_2Ac-Nle-OCH_3 (Lundblad & Stein, 1969; Chen & Tang, 1972). Again parallelism between renin and acid protease is apparent. Preliminary observations of inhibition of renin by aliphatic

diazo compounds (Inagami et al., 1974; McKown & Gregerman, 1975) and epoxides (Workman & Inagami, 1976), using crude preparations of hog and human renins, have been reported.

Treatment of renin with three different tyrosine-specific reagents, TNM, acetylimidazole, and DHT, has produced consistent results in which modification of 4 out of the total of 16 tyrosyl residues in a renin molecule is accompanied by virtually complete inactivation. Considerably higher concentrations of the reagents were required to modify additional tyrosyl residues, indicating higher accessibility of these four residues relative to the rest. Only two of the four residues seem to be located in the active site since the presence of pepstatin protects only two tyrosyl residues from the modification by TNM with concomitant preservation of most of the enzyme activity.

Although TNM is known to react with cysteinyl and methionyl residues as well as tyrosine, the loss of enzyme activity is not due to the modification of these residues since treatment of renin with iodoacetic acid and *N*-ethylmaleimide was shown to have no effect on renin activity (see Results). Similarly, inactivation of renin by acetylimidazole and DHT could be attributed solely to the modification of tyrosyl residues, since amino groups and histidyl residues, which are modified by these reagents, were shown not to be essential for renin activity in the modification studies using ethyl acetimidate (specific for amino group) and ethoxyformic anhydride (specific for both imidazole and amino groups). This view finds additional support in the observation that enzyme activity of acetylimidazole-inactivated renin (containing 4.2 residues of acetyltyrosine and 6.7 residues of *N*-acetyl groups per molecule) could be restored by hydroxylamine treatment through exclusive deacetylation of *O*-acetyl groups while leaving *N*-acetyl groups intact.

All of the arginyl residues of renin appear to be exposed since 8.6 of 9 arginyl residues in renin were modified by phenylglyoxal. While renin activity is completely lost by this modification, protection of 0.5–0.7 arginyl residue by pepstatin with concomitant preservation of enzyme activity indicates that one arginyl residue is located in the active site and presumably essential for the enzyme activity. Fliss & Viswanatha (1979) have reported that photosensitized reaction of the trimer of 2,3-butanedione with proteins results in the modification of α-amino groups, tryptophanyl residues as well as arginine residues. It is possible that phenylglyoxal may react with residues other than arginine residues under strong UV irritation; the amino acid analyses of phenylglyoxal-modified renin obtained in the present studies under dim room lighting gave good recovery of all residues other than arginine. Comparison of results obtained in the presence and absence of pepstatin again gave a difference only in arginine contents. Furthermore, modification of tryptophanyl residues by $HNBS(CH_3)_2Cl$ and of amino groups by ethyl acetimidate, ethoxyformic anhydride, and acetylimidazole was shown to be without effect on renin activity, indicating the absence of these residues in the active site.

In spite of certain similarities in the active site of renin and acid proteases, renin is distinct from the latter. It is one of the very few peptidases known to catalyze formation of peptide hormones from prohormones. Thus, its hydrolytic action is highly specific to one unique peptide bond present only in the prohormone angiotensinogen and is expressed in a neutral pH range. It has no general proteolytic activity (Misono et al., 1974).

The functional difference between renin and acid proteases should be accounted for by structural differences in the active sites. Complete inactivation of renin by the modification of arginyl residues observed in this study is in contrast to the only partial effect obtained in similar modification of arginyl residues in pepsin by Kitson & Knowles (1971) and Huang & Tang (1972). The present study did not include the modification of aliphatic hydrophobic amino acid residues and phenylalanine. Since the specificity determining structure of renin substrates consists of a number of aliphatic hydrophobic amino acid residues (Skeggs et al., 1968), it is highly likely that similar amino acid residues in the substrate binding site of renin confer the substrate specificity to this enzyme.

Tyrosyl residues have been implicated in the active site of pepsin (Perlman, 1966; Hollands & Fruton, 1968; Lockshina & Orekhovich, 1968; Kozlov et al., 1969). However, direct comparison with renin is not possible since detailed quantitative studies have not been reported with pepsin.

Characterization of the active site of renin had not been possible in the past due to the lack of pure preparation of renin. The mouse submaxillary gland enzyme was used in the present study because of its availability in relatively large quantities. This enzyme is closely related to mouse renal renin as reported by Michelakis et al. (1974). Thus, the results presented above represent the first detailed studies of the active site of renin which performs a very unique catalytic reaction.

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