

AFFINITY LABELING OF  
METALLOENDOPROTEASES

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AFFINITY LABELING OF  
METALLOENDOPROTEASES

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## SUMMARY

Thermolysin is irreversibly inhibited at pH 7.2 by the alkylating agents  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  and 2-(N-bromoacetyl-N-hydroxyamino)-4-methyl pentanonitrile. The inhibition reactions exhibit saturation kinetics with  $K_I$  values of 7.5 and 0.80 mM respectively. Competitive inhibitors of thermolysin (P-Leu-Trp-OK and Z-Phe-OH) hindered the alkylation. The stoichiometry of the reaction was demonstrated to be 0.97 to 1 by use of  $^{14}\text{C}$ -labeled  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$ . No change was observed in the amino acid analysis of the alkylated thermolysin. The inhibitor moiety could be removed with 1 mM NaOH or 1 M  $\text{NH}_2\text{OH}$ , observations which support the existence of an ester linkage between the enzyme and inhibitor. Degradation of thermolysin alkylated with  $^{14}\text{C}$ -labeled  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  with CN-Br demonstrated that the  $F_1$  fragment (residues 121-205) contained the inhibitor moiety. The evidence indicates that the inhibitor binds to the active site of thermolysin with interaction of the hydroxamic acid functional group with the active site zinc atom. Subsequent alkylation of Glu-143 irreversibly inactivates the enzyme. Other alkylating agents lacking the hydroxamic acid such as  $\text{BrCH}_2\text{CO-Phe-OCH}_3$ ,  $\text{BrCH}_2\text{CO-L-MeLeu-OCH}_3$ , and  $\text{BrCH}_2\text{CO-L-MeLeu-L-Ala-OCH}_3$  did not react with enzyme. The inhibitor  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  exhibited considerable specificity and would not inhibit the serine proteases chymotrypsin  $A_\alpha$  and subtilisin BPN', while the neutral metalloproteases A and B from B. subtilis were inactivated. Carboxypeptidase A was inactivated very slowly ( $t_{1/2} > 3$  days). Haloacetyl-N-hydroxy peptides with the appropriate amino acid or

peptide structures should be useful for the inhibition of other metallo-proteases.

A new method for the assay of the enzymatic activity of bacterial collagenase has been developed. The depsipeptide Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> is cleaved at the ester bond by clostridiopeptidase A. The enzymatic cleavage of the depsipeptide can be conveniently assayed by pH-stat titration. The K<sub>m</sub> value for Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> and clostridiopeptidase A was determined to be 1.3 ± 0.1 mM at pH 7.5. The cleavage is inhibited by 1,10-phenanthroline as well as by a series of hydroxamic acid peptides, but not by the serine protease inhibitor α-toluenesulfonyl fluoride.

## CHAPTER I

## INTRODUCTION

The development of inhibitors, in particular active site directed irreversible inhibitors, of the neutral protease thermolysin may be a significant step in the ultimate production of specific inhibitors of the biologically more important metalloendoproteases. An example is the enzyme collagenase which has been implicated in the debilitating disease arthritis (Harris and Krane, 1974). Collagenase has been found to participate in a variety of physiological processes, both beneficial and pathological. Specific irreversible inhibitors of these and other metalloendoproteases could be of potential therapeutic value, as well as aiding in the study of the active sites of these enzymes.

An essential requirement for the design of active site directed inhibitors of any enzyme is at least a limited knowledge of the substrate binding properties. This permits the logical design of inhibitors of the enzyme through the conservative modification of a substrate, resulting in a structure which will bind at the active site and interfere with catalysis. If enough detailed information is available on the mode of binding of substrates or inhibitors to the active site of an analogous model enzyme, then this knowledge can be applied to the synthesis of structures, not greatly resembling natural substrates, which may bind at the active site of a more complex enzyme and inhibit its catalytic activity. The design of inhibitors of mammalian collagenase appears best to

be approached through the latter method, owing to the complexity inherent in the triple helical structure of collagen, the enzyme's natural substrate.

To avoid the complications of using mammalian collagenase from the start and to gain information into the behavior of metalloendoproteases in general, a model enzyme (thermolysin) was chosen as our initial target for inhibition. Thermolysin (EC 3. 4. 24. 4) is a neutral endoprotease which requires a zinc atom at the active site for enzymatic activity. The amino acid sequence (Titani et al. 1977b) and the X-ray structure (Matthews et al., 1972a, b; Colman et al., 1972) of the enzyme have been determined. Typical chelating reagents such as ethylenediaminetetraacetic acid and 1,10-phenanthroline inhibit enzymatic activity. A series of potent active site directed reversible inhibitors containing the hydroxamic acid (Nishino and Powers, 1978) and phosphoramidate (Kam and Powers, unpublished results) functional groups have recently been synthesized.

Kester and Matthews (1977b) have compared in detail the X-ray structures of the active sites of carboxypeptidase A and thermolysin. The tetrahedral coordination around the zinc of carboxypeptidase A is more distorted than in thermolysin. The coordination of the carbonyl of the scissile peptide bond to the zinc atom is almost linear for thermolysin, while for carboxypeptidase A the same group is considerably tilted. There are significant differences between the positions of the catalytically important residues Glu-143 of thermolysin and Glu-270 of carboxypeptidase A relative to the scissile bond. Glu-270 is closer to the peptide bond cleaved than Glu-143. Two alternative mechanisms for the hydrolysis of peptide substrates by both enzymes have been proposed. In

the first mechanism, a glutamic acid (Glu-143), thermolysin, Glu-270, carboxypeptidase A) acts as a general base promoting the attack of a water molecule on the carbonyl carbon of the peptide bond cleaved, while in the second mechanism the glutamic acid acts as a nucleophile, attacking the carbonyl directly forming an anhydride subsequently cleaved by water. The structural similarity of the active sites of thermolysin and carboxypeptidase A suggests closely related mechanisms for both enzymes.

Kester and Matthews (1977b) determined through model building experiments, based on the mode of binding of dipeptides (1977a), that direct nucleophilic attack by Glu-143 on the carbonyl bond of the substrate is stereochemically implausible. The stereochemical restrictions observed for thermolysin are not present in the case of carboxypeptidase A, in which the carbonyl carbon of the pseudo-substrate glycyl-tyrosine binds  $1.4\text{\AA}$  closer to the glutamate oxygen than does an analogous dipeptide to thermolysin.

Weaver et al. (1977) proposed that the tight binding of the competitive inhibitor phosphoramidon (Suda et al., 1973; Komiyama et al., 1975) to thermolysin is the result of its being a tetrahedral transition state analog, very similar to the intermediate which would be formed by attack of a water molecule, promoted by Glu-143, on the carbonyl carbon of the scissile bond of a substrate. The observation that  $\text{CH}_3\text{-O-PO}_2\text{-Leu-NH}_2\text{\cdot K}$  ( $K_I$  in the mM range) is a significantly poorer inhibitor of thermolysin than is  $\text{PO}_3\text{-Leu-NH}_2\text{\cdot 2K}$  ( $K_I$  in the  $\mu\text{ M}$  range) (Kam and Powers, unpublished results) argues against these inhibitors being simply transition state analogues.

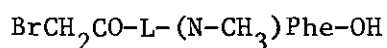
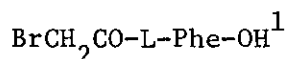
Evidence for the general base mechanism for carboxypeptidase A hydrolysis of peptide substrates has been provided by Breslow and Wernick (1976, 1977) through oxygen-18 exchange studies; however, they do propose that ester cleavage by this enzyme is through anhydride formation.

The active site His-231 of thermolysin has been suggested (Kester and Matthews, 1977a; Weaver et al., 1977) to assist peptide cleavage by donating a proton to the leaving amino group. Model building experiments (Kester and Matthews, 1977b) indicate that in the transition state the newly tetrahedral nitrogen of the scissile bond moves to within about  $3.5\text{\AA}$  of  $\overset{\circ}{\text{N}}_{\text{e}2}$  of His-231. A small movement ( $0.5\text{\AA}$ ) of His-231 would then bring the imidazole into the expected position for direct proton transfer. However, proton transfer via a water molecule cannot be ruled out. The role of the active site Tyr-248 of carboxypeptidase A in catalysis remains uncertain. This residue appears to be the only candidate for proton donation at the active site of carboxypeptidase A analogous to His-231 of thermolysin.

From structural considerations alone, the close positional correspondence of the zinc atoms and the respective glutamic acid residues of both enzymes suggest that this stereochemical juxtaposition is vital to catalysis. There appears to be no absolute requirement for the involvement of a histidine or a tyrosine in hydrolysis catalyzed by these two proteases.

In the design of an active site directed irreversible inhibitor (an affinity label) of thermolysin, the considerations discussed above concerning the active sites of two zinc containing proteases were valuable guides as to where an alkylating functional group should be

located in a potential inhibitor with a reasonable expectation of labeling either the Glu-143 or His-231 residues. A lead in this direction appeared to be the compounds used by Hass and Neurath (1971a, b) to label the Glu-270 of carboxypeptidase A. Carboxypeptidase B is



modified by N-Bromoacetyl-D-arginine, a reagent that stoichiometrically reacts with the active site glutamic acid residue (Plummer, 1971; Kimmel and Plummer, 1972, Sokolovsky and Zisapel, 1974). The side chains of Tyr-248 and a methionine residue of carboxypeptidase B are modified respectively by bromoacetamido-butylguanidine (Plummer, 1969) and N-bromoacetyl-p-aminobenzyl-L-succinic acid (Zisapel and Sokolovsky, 1974). Since thermolysin and carboxypeptidase A have similar primary substrate binding requirements for a bulky hydrophobic residue occupying the  $S_1'$

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<sup>1</sup>Abbreviations used:  $\text{BrCH}_2\text{CO-Phe-OCH}_3$ , N-bromoacetyl-L-phenylalanine methyl ester;  $\text{BrCH}_2\text{CO-L-MeLeu-OCH}_3$ , N-bromoacetyl-L-N-methylleucine methyl ester;  $\text{BrCH}_2\text{CO-L-MeLeu-L-Ala-OCH}_3$ , (N-bromoacetyl-L-N-methylleucyl-L-alanine methyl ester;  $\text{ClCH}_2\text{CO-(N-OH)Leu-OCH}_3$ , N-chloroacetyl-DL-N-hydroxyleucine methyl ester; DMF, dimethylformamide; FA-Gly L-Leu-NH<sub>2</sub>, furylacryloylglycyl-L-leucinamide; Hepes, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; P-Leu-Trp-OK, N-Phosphoryl-L-leucyl-L-tryptophan tripotassium salt; Pipes, piperazine-N, N-bis(ethanesulfonic acid); Tris, tris (hydroxymethyl) aminomethane.

(notation of Schechter and Berger, 1967) subsite, modification of the inhibitors described by Hass and Neurath (thermolysin does not "like" a free carboxyl group at the  $P_1'$  site) were examined as possible affinity labels of thermolysin with disappointing results.

This thesis reports the unsuccessful attempt to extend the class of compounds developed by Hass and Neurath as possible inhibitors of the metalloendoprotease thermolysin, and the successful utilization of haloacetyl-N-hydroxy derivatives of amino acids as affinity labels of thermolysin and the B. subtilis neutral proteases A and B. The results of this work has been reported elsewhere (Rasnick, 1978; Rasnick and Powers, 1978). Potentially, these structures could be incorporated into specific active site directed irreversible inhibitors of the bacterial and mammalian collagenases.

In order to determine the effectiveness of the inhibitors synthesized for use with collagenase, a method for the convenient and reliable assay of the enzyme's activity is desirable. Unfortunately, there is no such method at present for the assay of the mammalian enzyme. The assay of bacterial collagenase (Clostridiopeptidase A) activity, using the commercially available substrate 4-phenylazobenzoyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine, is cumbersome and not very sensitive. As a result of the close relationship between the efficient development of inhibitors and routine and reliable enzyme assays, we include a report of a new method for the assay of bacterial collagenase enzymatic activity.

## CHAPTER II

## MATERIALS AND METHODS

The following enzymes were obtained from the Sigma Chemical Company: thermolysin purified as described by Titani et al. (1972a); carboxypeptidase A used as an aqueous suspension containing a trace of toluene; subtilisin BPN' Type VII was used without further purification; Clostridiopeptidase A Type VI. Crystalline chymotrypsin A<sub>α</sub> (Worthington Biochemical Corp.) was also used without further purification. A mixture of neutral proteases A and B from B. subtilis was kindly provided by Dr. J. Feder of Monsanto and separated and purified by Dr. Norikazu Nishino on an affinity column (HONHCOCH (CH<sub>2</sub>C<sub>6</sub>H<sub>5</sub>)CO-Ala-Gly-Affi-Gel 101, Affi-Gel 101 was supplied by Bio-Rad) in our laboratory.

Other commercial reagents of the highest quality available were obtained as follows: chloroacetic acid, glycolic acid, and bromoacetyl bromide (Aldrich Chemical Co.); furylacryloylglycyl-L-leucinamide (FA-Gly-Leu-NH<sub>2</sub>) (Vega-Fox Biochemicals); N-t-butyloxycarbonyltyrosine-p-nitrophenyl ester (Boc-Tyr-ONp) (Bachem Fine Chemicals); N-carbobenzyloxy-glycylphenylalanine (Z-Gly-Phe-OH) and Z-Phe-OH (Pierce Chemical Co.); (1-<sup>14</sup>C) chloroacetic acid (New England Nuclear); cyanogen bromide (Eastman Organic Chemicals) was stored in a sealed desiccator at 4° when not in use. L-Phenylalanine methyl ester hydrochloride was synthesized by Dr. R. Boone in our laboratory. Sephadex G-75, medium, was purchased from the Sigma Chemical Company. Sephadex G-25, fine, was obtained from

Pharmacia Fine Chemicals and Bio-Gel P-100 (100-200 mesh) from Bio-Rad Laboratories. N-Phosphoryl-L-leucyl-L-tryptophan tripotassium salt (P-Leu-Trp-OK) and N-phosphoryl-L-isoleucyl-L-alanine tripotassium salt (P-Ile-Ala-OK) were synthesized by Dr. Chih-Min Kam of our group. Z-Gly-Pro-Leu-NHOH, Gly-Pro-Leu-(N-OH)Gly-ProOH, Boc-Gly-Pro-Leu-(N-OH)Gly-ProOH, HONHCOCH<sub>2</sub>CO-ProNH<sub>2</sub>, Boc-Gly-Pro-Leu-(N-OH)Gly-OCH<sub>2</sub>CH<sub>3</sub>, Z-Gly-Pro-Leu-OH, Z-Gly-Gly-LeuNHOH, Z-Gly-LeuNHOH, HSCH<sub>2</sub>CH(CH<sub>2</sub>C<sub>6</sub>H<sub>5</sub>)CO-Ala-GlyNH<sub>2</sub>, and HONHCOCH(*i*-Bu)CO-Ala-GlyNH<sub>2</sub> were synthesized by Dr. Norikazu Nishino of our group.

Analytical Procedures. Peptidase activity of thermolysin and neutral proteases A and B were determined by following the decrease in absorption at 345 nm due to the enzymatic hydrolysis of a 2 ml solution of FA-Gly-Leu-NH<sub>2</sub> (1.2 mM) as described by Walsh et al. (1975) with the exception that Tris buffer was used in place of Hepes. Protein concentrations were determined by measuring the absorption at 280 nm using a Beckman 25 thermostated spectrophotometer. A molar absorptivity of 66,400 M<sup>-1</sup> cm<sup>-1</sup> was used for thermolysin (Ohta et al., 1966; Titani et al., 1972b). The concentrations of neutral proteases A and B, and chymotrypsin A<sub>α</sub> were similarly determined,  $\epsilon_{280}^{1\%} = 14.8$  and  $14.7 \text{ cm}^{-1}$  (Walsh et al., 1975) and  $\epsilon_{282}^M = 50,000 \text{ M}^{-1} \text{ cm}^{-1}$  (Wilcox, 1970) respectively. The concentration of clostridiopeptidase A was estimated using  $\epsilon_{280}^{1\%} = 18.0$  (Worthington Enzyme Manual, 1972).

Radioactivity was measured with a Beckman LS-100C liquid scintillation spectrometer. An efficiency of 52% was observed for 1 ml aqueous samples dispersed in 10 ml of Triton X-100 (Lieberman and Moghissi, 1970). Elemental analysis was performed by Atlantic Microlab, Atlanta, Georgia.

Infrared spectra were measured on a Perkin Elmer 457 instrument. Proton magnetic resonance (pmr) spectra were taken on a Varian T-60 instrument. Quantitative amino acid analysis was performed in the laboratory of Dr. J. Travis (University of Georgia) on 24 hr. hydrolysates (6N HCl). Thin layer chromatography was performed on Merck silica gel G plates with the solvent systems;  $R_f^1$ , chloroform-methanol (10:1, v/v),  $R_f^2$ , cyclohexane-ethyl acetate (1:3, v/v),  $R_f^3$ , chloroform-methanol (5:1, v/v);  $R_f^4$ , n-butanol-acetic acid-water (4:1:1, v/v).

N-Bromoacetyl-L-phenylalanine methyl ester. To a suspension of L-phenylalanine methyl ester hydrochloride (10.0 g, 46.5 mmol) in 250 ml of ethyl acetate were added bromoacetyl bromide (9.40 g, 46.5 mmol) and triethylamine (9.40 g, 93.0 mmol) at  $-10^\circ$  with stirring 30 min at  $-5^\circ$ . The reaction was quenched with water (200 ml). The organic phase was separated and washed with 100 ml of 1.0 M HCl, 100 ml of 0.1 M  $\text{NaHCO}_3$ , and 100 ml of water and then dried over anhydrous magnesium sulfate. The ethyl acetate was removed by evaporation to give a deep red oil which was taken up in 30 ml of ether. Crystals formed following the addition of petroleum ether. Yield was 2.90 g (28%), mp  $83-84^\circ$ ,  $R_f^1$  0.92,  $R_f^2$  0.77. This compound had been prepared by a different method, mp  $80-82^\circ$  (Williams, 1952).

The pmr ( $\text{CDCl}_3$ ) spectrum showed peaks at  $\delta$  7.2 (6H, s, Ph + NH), 4.8 (1H, m, N-CH-), 3.8 (2H, s,  $\text{BrCH}_2$ -), 3.7 (3H, s,  $\text{CH}_3$ -O), 3.2 (2H, d, Ph-CH<sub>2</sub>-).

N-Bromoacetyl-L-N-methylleucine methyl ester. To a suspension of L-N-methylleucine methyl ester hydrobromide (410 mg, 1.7 mmol) (prepared by treating N-carbobenzyloxy-L-N-methylleucine methyl ester (Okamoto *et*

al., 1974) with a 30% solution of HBr in acetic acid) in 50 ml of ethyl acetate were added bromoacetyl bromide (350 mg, 1.7 mmol) and N-methylmorpholine (350 mg, 3.4 mmol) at room temperature with stirring 24 hr. Water (50 ml) was then added and the organic phase was separated and washed with 50 ml of 1.0 M HCl, 50 ml of 0.1 M NaHCO<sub>3</sub>, and 50 ml of water and then dried over anhydrous magnesium sulfate. The ethyl acetate was removed by evaporation to give a colorless oil 270 mg (57%). The ir (neat) had absorption bands at 1740 (ester CO) and 1660 cm<sup>-1</sup> (amide CO).

The pmr (CDCl<sub>3</sub>) spectrum showed peaks at δ5.2 (1H, t, N-CH-), 4.0 (2H, s, BrCH<sub>2</sub>), 3.7 (3H, s, CH<sub>3</sub>-O), 3.0 (3H, s, CH<sub>3</sub>N), 2.0-1.2 (3H, m, CH<sub>2</sub>CH), 0.9 (6H, d, CH(CH<sub>3</sub>)<sub>2</sub>).

Anal. calcd for C<sub>10</sub>H<sub>18</sub>BrNO<sub>3</sub>: C, 42.87; H, 6.48; N, 5.00 Found: C, 42.75; H, 6.34; N, 5.21.

N-Bromoacetyl-L-N-methylleucyl-L-alanine methyl ester was similarly prepared by treating L-N-methylleucyl-L-alanine methyl ester hydrobromide (192 mg, 0.62 mmol) with bromoacetyl bromide (125 mg, 0.62 mmol) to give 140 mg (62%) of an oil.

The pmr (CDCl<sub>3</sub>) spectrum showed peaks at δ6.9 (1H, d, HN), 5.2 (1H, t, NCHCH<sub>2</sub>), 4.4 (1H, m NHCHCH<sub>3</sub>), 4.0 (2H, s, BrCH<sub>2</sub>), 3.8 (3H, s, CH<sub>3</sub>-O), 3.0 (3H, s, CH<sub>3</sub>-N), 1.7 (3H, m CH<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 1.2 (3H, d, NHCHCH<sub>3</sub>), 0.9 (6H, d, (CH<sub>3</sub>)<sub>2</sub>).

2-(N-Bromoacetyl-DL-N-hydroxyamino)-4-methylpentanonitrile. To DL-2-hydroxyamino-4-methylpentanonitrile (1.73 g, 13.5 mmol) (Neelakantan and Hartung, 1958) in 20 ml of acetonitrile was added bromoacetyl bromide (1.36 g, 6.75 mmol) at room temperature. The reaction was stopped after 2 hr. The acetonitrile was removed by evaporation and the residue was

dissolved in 100 ml of chloroform, washed with 50 ml of 0.1 M  $\text{NaHCO}_3$ , 50 ml 1.0 M  $\text{HCl}$ , and 50 ml water then dried over anhydrous magnesium sulfate. The  $\text{CHCl}_3$  was removed by evaporation to leave 1.28 g of a yellow oil. The oil was chromatographed on 50 g of silica gel 60 and eluted with 0.5% ethyl acetate in chloroform to give 830 mg (50%) of a colorless oil which produced an intense red color when treated with 10% methanolic ferric chloride. The oil decomposed upon attempted distillation at  $120^\circ$  and 0.05 mm pressure. The ir (neat) had absorption bands at 3220 (OH), 2250 (CN), and  $1655\text{ cm}^{-1}$  (amide CO);  $R_f^1$  0.80,  $R_f^2$  0.55.

The pmr ( $\text{CDCl}_3$ ), spectrum showed peaks at  $\delta$ 5.6 (1H, t,  $-\text{CH}-\text{CN}$ ), 4.2 (2H, s,  $\text{BrCH}_2-$ ), 2.2-1.5 (3H, m,  $\text{CH}_2\text{CH}$ ), 0.9 (6H, d,  $\text{CH}(\text{CH}_3)_2$ ).

N-Chloroacetyl-DL-N-hydroxyleucine methyl ester was prepared by the procedure of Cook and Slater (1956); mp  $80-80.5^\circ$  (lit. mp  $81^\circ$ ).

The pmr ( $\text{CDCl}_3$ ) spectrum showed peaks at  $\delta$ 5.3 (1H, t, N-CH), 4.4 (2H, s,  $\text{ClCH}_2-$ ), 3.8 (3H, s,  $\text{CH}_3-\text{O}$ ), 2.2-1.4 (3H, m,  $\text{CH}_2\text{CH}$ ), 0.9 (6H, d,  $\text{CH}(\text{CH}_3)_2$ ).

Anal. calcd for  $\text{C}_9\text{H}_{16}\text{ClNO}_4$ : C, 45.48; H, 6.78; N, 5.89. Found: C, 45.50; H, 6.82; N, 5.88.

$\alpha$  - Bromoisocaproylhydroxamic acid. To  $\alpha$  - bromoisocaproic acid (1.0 g, 5.1 mmol) at  $0^\circ$  were added DCC(1.1 g, 5.1 mmol) and N-hydroxysuccinamide (0.59 g, 5.1 mmol) in 50 ml dioxane. The mixture was stirred 1 hr at  $0^\circ$  and 2 hr at room temperature. The DCU was filtered off and to the filtrate was added 1 equivalent of  $\text{NH}_2\text{OH}$  in 75 ml DMF. This solution was stirred 10 hr at room temperature. The dioxane and DMF were evaporated off to give an oil which turned a 10% solution of methanolic ferric chloride red. The oil was crystallized from ether-petroleum

ether to give 537 mg (50%), mp 121-122°.

Anal. calcd for  $C_6H_{12}BrNO_2$ : C, 34.31; H, 5.76; N, 6.67. Found: C, 34.36; H, 5.79; N, 6.66.

N-(3-methyl-1-methoxycarbonylbutyl)-3-isobutyl-oxaziridine. To isovaleriminoleucine methyl ester (2.09 g, 9.8 mmol) in 5 ml  $CH_2Cl_2$  was added m-chloroperbenzoic acid (85%) (1.69 g, 9.8 mmol) in 15 ml  $CH_2Cl_2$  in portions with cooling to  $-5-0^\circ$  over 1.5 hr, then stirred overnight at room temperature. The solution was filtered and the filtrate was washed with 20 ml of 1.0 M HCl, 20 ml of saturated  $NaHCO_3$  solution, and 20 ml of water, then dried over anhydrous magnesium sulfate. The  $CH_2Cl_2$  was evaporated off to give 2 g (89%) of an oil. The oil was purified by distillation, bp 82-83° (0.08 mm)

The nmr (neat) showed peaks at  $\delta$ 3.9 (1H, t, N-CH-), 3.7 (3H, s,  $OCH_3$ ), 2.8 (1H, m,  $\sqrt{\frac{O}{N}}$ ), 2.2-1.2 (6H, m,  $2(CH_2-CH)$ ), 0.9 (12H, m,  $2(CH_3)_2$ ). The compound oxidized iodide to iodine in acetic acid.

Anal. calcd for  $C_{12}H_{23}NO_3$ : C, 62.85; H, 10.11; N, 6.11. Found: C, 62.90; H, 10.08; N, 6.12.

N-Benzylidene-DL-leucine-N-oxide methyl ester. Anti-benzaldoxime (2.02 g, 16.7 mmol) (Polonski and Chimiak, 1976) and DL- $\alpha$ -bromoisocaproic acid methyl ester (3.5 g, 16.7 mmol) were added to a 20 ml solution of methanol containing sodium (0.38 g, 16.7 mmol). The solution was stirred overnight at room temperature. The methanol was evaporated off and the residue was treated with 15 ml water and the pH was adjusted to 7 with 0.1 M HCl. The aqueous solution was extracted with 20 ml of chloroform. The organic layer was dried over anhydrous magnesium sulfate. The chloroform was evaporated off and the residue was crystallized from ether to

give 1.0 g (24%), mp 106-108°.

The nmr ( $\text{CDCl}_3$ ) showed peaks at  $\delta$ 8.2 (2H, m Ph-H), 7.5 (4H, m, Ph-CH=), 4.8 (1H, q, N-CH-), 3.8 (3H, s,  $\text{OCH}_3$ ), 2.5-1.3 (3H, m  $\text{CH}_2\text{CH-}$ ), 0.9 (6H, d,  $\text{C}(\text{CH}_3)_2$ ).

Anal. calcd for  $\text{C}_{14}\text{H}_{19}\text{NO}_3$ : C, 67.45; H, 7.68; N, 5.62. Found: C, 67.39; H, 7.69; N, 5.63.

N-Carbobenzyloxy-glycyl-L-proline. To N-carbobenzyloxyglycine (14.9 g, 71.5 mmol) in methanol (150 ml) at  $-5^\circ$  was added pyridine (5.65 g, 71.5 mmol) and thionyl chloride (8.51 g, 71.5 mmol). The reaction mixture was stirred 30 min at  $0^\circ$  followed by the addition of proline (8.22 g, 71.5 mmol) in 50 ml methanol and pyridine (5.65 g, 71.5 mmol) at  $0^\circ$ . The reaction mixture was allowed to warm to room temperature with stirring 1 hr more. The methanol was evaporated off and the residue was crystallized from ethyl acetate to give 6.9 g (31.5%), mp 154-155° (Lit. mp 155-157°, Goodman and Stueben, 1962).

Glycolyl-L-prolinamide. To glycolic acid (1.52 g, 20 mmol), proline methyl ester hydrochloride (3.63 g, 22 mmol), N-hydroxybenzotriazole (0.27 g, 2 mmol), and triethylamine (3.1 ml, 22 mmol) in 100 ml THF at  $0^\circ$  was added DCC (4.12 g, 20 mmol) and stirred 1 hr at  $0^\circ$ , then set aside 12 hr at  $4^\circ$ , followed by stirring 1 hr at room temperature. The DCU and triethylamine hydrochloride were removed by filtration and the THF of the filtrate was removed by evaporation. The residue was treated with ethyl acetate (30 ml) and filtered. The ethyl acetate was removed by evaporation to give an oil which was treated with methanol saturated with ammonia (100 ml) and allowed to stand at room temperature for 20 hr. The methanol and ammonia were removed by evaporation leaving an oil which was

crystallized from methanol-ether to give 1.82 g (53%), mp 145-146°. The ir (nujol) had peaks at 3420 and 3100 (OH and HN stretch) and 1640  $\text{cm}^{-1}$  (amide CO);  $R_f^3$  0.26.

The nmr (DMSO- $d_6$ ) had peaks at  $\delta$ 7.2-6.6(2H, d,  $\text{NH}_2$ ), 4.5-3.6 (3H, m,  $\text{HO}-\underline{\text{CH}_2}-\text{N}-\underline{\text{CH}}$ ), 3.3 (3H, m,  $\text{N}-\underline{\text{CH}_2}+\underline{\text{OH}}$ ), 1.8 (4H, m,  $-\underline{\text{CH}_2}-\underline{\text{CH}_2}-\underline{\text{CH}_2}-\text{N}$ ).

Anal. calcd for  $\text{C}_{17}\text{H}_{22}\text{N}_2\text{O}_3$ : C, 48.83; H, 7.02; N, 16.27. Found: C, 49.04; H, 7.11; N, 16.42.

N-t-Butyloxycarbonyl-L-leucyl-glycolyl-L-prolinamide was prepared by the method of Shimohigashi *et al.* (1977) to give 1.44 g (49%). The compound was used without characterization.

N-Carbobenzyloxy-glycyl-L-prolyl-L-leucyl-glycolyl-L-prolinamide (Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub>). To N-carbobenzyloxy-glycyl-L-proline (1.53 g, 5.0 mmol) in 20 ml THF at -20° was added N-methylmorpholine (0.50 g, 5.0 mmol) and isobutylchloroformate (0.58 g, 5.0 mmol). After stirring 5 min at -20° L-leucyl-glycolyl-L-prolinamide hydrochloride (1.6 g, 5.0 mmol) (prepared by treating N-t-butyloxycarbonyl-L-leucyl-glycolyl-L-prolinamide with 2.7 M HCl in dioxane at room temperature for 30 min followed by evaporation of the HCl and dioxane) in 15 ml DMF and N-methylmorpholine (0.50 g, 5.0 mmol) were added at -20°. The mixture was stirred 1 hr at -20° and overnight at room temperature. The reaction was filtered and the filtrate was evaporated to a residue which was taken up in 40 ml chloroform followed by washing with saturated  $\text{NaHCO}_3$  solution (15 ml), and water (15 ml), then dried over anhydrous magnesium sulfate. The chloroform was removed by evaporation and the residue was crystallized from ethyl acetate-ether to give 1.4 g (49%) mp 109-113°;  $R_f^3$  0.65.

The nmr ( $\text{CDCl}_3$ ) showed peaks at  $\delta$ 7.3 (6H, s, Ph-H+NH), 6.8 (1H, s, NH), 5.9 (2H, s,  $\text{NH}_2$ ), 5.1 (2H, s, Ph- $\text{CH}_2$ ), 4.6 (5H, m, 3(N- $\text{CH}$ )+ $\text{OCH}_2$ ), 4.0 (2H, d, NH- $\text{CH}_2$ ), 3.5 (4H, m, 2(-N- $\text{CH}_2$ ), 2.4-1.5 (11H, m, 2(-NCH- $\text{CH}_2\text{CH}_2$ )+ $\text{CH}_2\text{CH}(\text{CH}_3)_2$ ), 0.9 (6H, d,  $\text{CH}(\text{CH}_3)_2$ ).

Anal. calcd for  $\text{C}_{28}\text{H}_{39}\text{N}_5\text{O}_8$ ; C, 58.63; H, 6.85; N, 12.21. Found: C, 58.21; H, 6.94; N, 12.08.

N-t-Butyloxycarbonyl-glycyl-L-prolinamide. To N-t-butyl-oxycarbonyl-glycine (9.26 g, 53 mmol) in 175 ml THF at  $-20^\circ$  was added N-methylmorpholine (5.35 g, 53 mmol) and isobutylchloroformate (7.26 g, 53 mmol). After stirring 5 min at  $-20^\circ$  proline methyl ester hydrochloride (8.75 g, 53 mmol) in 20 ml chloroform and N-methylmorpholine (5.35 g, 53 mmol) were added at  $-20^\circ$ . The reaction was allowed to reach room temperature (about 20 min). The mixture was filtered and the filtrate was evaporated to a residue which was taken up in 100 ml chloroform, washed with 50 ml of saturated  $\text{NaHCO}_3$  solution and 50 ml of water, then dried over anhydrous magnesium sulfate. The chloroform was evaporated off and the residue was taken up in 150 ml of methanol saturated with ammonia and allowed to stand 2 days at room temperature. The methanol and ammonia were removed by evaporation to leave a residue which was crystallized from 100 ml ether to give 3.8 g (26.5%), mp  $145-147^\circ$ .

The nmr ( $\text{CDCl}_3$ ) showed peaks at  $\delta$ 7.0 (1H, s, NH), 6.1 (1H, s, NH), 5.7 (1H, t,  $\text{NH}-\text{CH}_2$ ), 4.6 (1H, t, -N- $\text{CH}-\text{CH}_2$ ), 4.0 (2H, d, NH- $\text{CH}_2$ ), 3.6 (2H, m, -N- $\text{CH}_2$ -), 2.2 (4H, m, -N- $\text{CH}_2\text{CH}_2\text{CH}_2$ ), 1.5 (9H, s,  $\text{O}-\text{C}(\text{CH}_3)_3$ ).

N-t-Butyloxycarbonyl-L-leucyl-glycyl-L-prolinamide. To N-t-butyl-oxycarbonyl-L-leucine (1.28 g, 5.5 mmol) in 50 ml THF at  $-20^\circ$  were added N-methylmorpholine (0.56 g, 5.5 mmol) and isobutylchloroformate (0.76 g,

5.5 mmol). After stirring 5 min. at  $-20^{\circ}$  glycyl-L-prolinamide (1.5 g, 5.5 mmol) (prepared by treating N-t-butyloxycarbonyl-glycyl-L-prolinamide with HCl-dioxane for 30 min followed by evaporation of the HCl and dioxane) in 40 ml DMF and N-methylmorpholine (0.56 g, 5.5 mmol) were added at  $-20^{\circ}$ . The reaction was allowed to warm and stand at room temperature overnight. The mixture was filtered and the filtrate was evaporated to a residue which was taken up in 80 ml chloroform and washed with 20 ml saturated  $\text{NaHCO}_3$  solution and 20 ml water, then dried over anhydrous magnesium sulfate. The chloroform was evaporated off and the residue was crystallized from 100 ml ether to give 0.97 g (46%), mp  $132.5\text{--}134^{\circ}$ .

The nmr ( $\text{CDCl}_3$ ) had peaks at  $\delta$ 7.6 (1H, s, NH), 7.4 (1H, s, NH), 6.7 (1H, s, NH), 6.3 (1H, m,  $\text{NH-CH}_2$ ), 4.5 (2H, m,  $-\text{N-CH-CH}_2\text{-NH-CH-CH}_2$ ), 4.1 (2H, d,  $\text{NH-CH}_2$ ), 3.6 (2H, m,  $-\text{N-CH}_2$ ), 2.1 (4H, m,  $-\text{N-CH}_2\text{-CH}_2\text{-CH}_2-$ ), 1.7 (3H, m,  $\text{CH}_2\text{CH}$ ), 1.4 (9H, s,  $\text{O-C(CH}_3)_3$ ), 0.9 (6H, d,  $\text{CH(CH}_3)_2$ ).

N-Carbobenzyloxy-glycyl-L-prolyl-L-leucyl-glycyl-L-prolinamide (Z-Gly-Pro-Leu-Gly-ProNH<sub>2</sub>). To N-carbobenzyloxy-glycyl-L-proline (0.40 g, 1.3 mmol) in 20 ml DMF at  $-12^{\circ}$  were added N-methylmorpholine (150  $\mu$ l) N-hydroxybenzotriazole (18 mg, 0.13 mmol), L-leucyl-glycyl-L-prolinamide hydrochloride (0.42 g, 1.3 mmol) and DCC (0.27 g, 1.3 mmol). The reaction was stirred 4 hr at  $0^{\circ}$  and overnight at room temperature. The mixture was filtered, and the filtrate was evaporated to a residue which was treated with 20 ml water and filtered. The filtrate was treated with Rexyn I-300 for 1.5 hr and filtered. The water was removed by evaporation and the residue was pumped dry over  $\text{P}_2\text{O}_5$  to give 0.144 g (19.5%) mp  $201\text{--}206^{\circ}$ . Recrystallized from methanol-ether, mp  $209\text{--}211^{\circ}$ .

The nmr (DMSO- $d_6$ ) showed peaks at  $\delta$ 7.4 (5H, s, Ph-H), 7.2-6.9 (5H, d, 3 (NH)+NH<sub>2</sub>), 5.1 (2H, s, Ph-CH<sub>2</sub>), 4.3 (2H, s, 2(-N-CH-), 3.9 (4, m, 2(NH-CH<sub>2</sub>), 3.7-3.2 (5H, m, 2(-N-CH<sub>2</sub>)+NH-CH-), 1.9 (8H, m, 2(-N-CH<sub>2</sub>CH<sub>2</sub>-CH<sub>2</sub>), 1.5 (3H, m, CH<sub>2</sub>CH), 0.9 (6H, s, CH(CH<sub>3</sub>)<sub>2</sub>).

Anal. calcd for C<sub>28</sub>H<sub>40</sub>N<sub>6</sub>O<sub>7</sub>: C, 58.73; H, 7.05; N, 14.68. Found: C, 58.99; H, 7.01; N, 14.69.

N-(1-<sup>14</sup>C) Chloroacetyl-DL-N-hydroxyleucine methyl ester. (1-<sup>14</sup>C)

Chloroacetic acid (2.3 mg, 41.8 mCi/mmol) and unlabeled chloroacetic acid (160 mg, 1.7 mmol) were dissolved in 1.5 ml of dry THF. To this solution at -5° were added pyridine (150  $\mu$ l) and thionyl chloride (150  $\mu$ l). The reaction was protected with a calcium chloride drying tube and allowed to proceed 1 hr at room temperature. The solution was filtered and to the filtrate at 0° were added 200 mg of solid NaHCO<sub>3</sub> and DL-N-hydroxyleucine methyl ester (410 mg, 2.55 mmol) (Cook and Slater, 1956) in 3 ml of dry THF. The solution was allowed to stand 30 min at room temperature. Chloroform (25 ml) was added to the reaction solution followed by washing with 15 ml of saturated NaHCO<sub>3</sub> solution, 15 ml 1.0 M HCl, and 15 ml water then dried over anhydrous magnesium sulfate. The THF and chloroform were removed by evaporation to leave an oil which was taken up in 5 ml ether. Crystallization was initiated by the addition of petroleum ether. The overall yield was 196 mg (49%); mp 80-80.5°. The compound produced an intense red color when treated with 10% methanolic ferric chloride. The measured specific activity was 0.53 mCi/mmol.

Treatment of Thermolysin with BrCH<sub>2</sub>CO-Phe-OCH<sub>3</sub>, BrCH<sub>2</sub>CO-L-MeLeu-OCH<sub>3</sub> and BrCH<sub>2</sub>CO-L-MeLeu-L-Ala-OCH<sub>3</sub>. The procedures for treating thermolysin with all of the alkylating agents were the same. To a 1 ml solution

of the enzyme (0.10 M Tris, 0.01 M  $\text{CaCl}_2$ , pH 7.2) at 25° was added 50  $\mu\text{l}$  of a dimethylformamide (DMF) solution of the alkylating agent. The final concentrations were 0.19  $\mu\text{M}$  and 0.10 mM for the enzyme and alkylating agent respectively. Incubation was continued at least 48 hr. Aliquots (50  $\mu\text{l}$ ) of the incubating solution were periodically assayed for enzyme activity. A solution containing the enzyme but no alkylating agent was treated similarly.

Treatment of Thermolysin with 2-(N-Bromoacetyl-N-hydroxamino)-4-methylpentanonitrile. To 1 ml solutions of thermolysin in the above Tris buffer at 25° were added 50  $\mu\text{l}$  of DMF solutions of inhibitor at the appropriate concentrations. The final inhibitor concentrations ranged from 0.87 to 0.12 mM, the enzyme concentration was 0.30  $\mu\text{M}$ , and the final DMF concentration was 5%. Aliquots (50  $\mu\text{l}$ ) from each incubating solution were periodically assayed for residual enzyme activity and compared with a control enzyme solution lacking the inhibitor.

Treatment of Thermolysin and the Neutral Proteases A and B with  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$ . To 1 ml solutions of the appropriate enzyme at 25° were added 50  $\mu\text{l}$  of DMF solutions of inhibitor at the appropriate concentrations. The final inhibitor concentrations with thermolysin ranged from 10.5 to 2.1 mM; for neutral proteases A and B the concentration of the inhibitor was 8.4 mM. The final concentration of thermolysin was 5.4  $\mu\text{M}$ ; and for neutral proteases A and B the final concentrations were 0.67 and 0.31 mg/ml respectively.

The amino acid analyses for both purified alkylated and nonalkylated thermolysin were performed after chromatography on Sephadex G-25.

Treatment of Thermolysin with  $\alpha$ -Bromoisocaproylhydroxamic acid.

A thermolysin solution (1 ml) was treated similarly with 50  $\mu$ l of a DMF solution of  $\alpha$ -bromoisocaproylhydroxamic acid to give final enzyme and inhibitor concentrations of 5.4  $\mu$ M and 10 mM respectively. The incubation proceeded 3 days.

Treatment of Thermolysin with N-(3-methyl-1-methoxycarbonylbutyl)-3-isobutyl-oxaziridine and N-Benzylidene-DL-leucine-N-oxide methyl ester. A thermolysin solution (1 ml) was again treated similarly with 50  $\mu$ l of a DMF solution of the appropriate inhibitor to give a final enzyme concentration of 5.0  $\mu$ M and inhibitor concentrations of 2 mM each. Incubation proceeded 3 days.

Treatment of Carboxypeptidase A with  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$ .

To a 1 ml solution of carboxypeptidase A (0.46 mg/ml, 1.0 M NaCl, 5 mM  $\text{K}_2\text{HPO}_4$ , pH 7.5) was added 50  $\mu$ l of a methanolic solution of the alkylating agent to give a final inhibitor concentration of 1.0 mM. This solution was incubated at 25° for 3 days along with a control solution containing the enzyme and 50  $\mu$ l of methanol. Periodically 50  $\mu$ l aliquots were assayed for residual enzyme activity by following the hydrolysis of a 2 ml solution of Z-Gly-Phe (1.2 mM, 0.45 M KCl, 0.05 M Tris, pH 7.5) spectrophotometrically at 223 nm.

Treatment of Chymotrypsin  $A_\alpha$  and Subtilisin BPN' with  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$ . A 1 ml solution of chymotrypsin  $A_\alpha$  (0.10 M HEPES, 1.0 M NaCl, pH 7.5) and 50  $\mu$ l of a methanolic solution of the alkylating agent were mixed together to give final enzyme and alkylating agent concentrations of 0.93  $\mu$ M and 1.0 mM respectively. This solution was incubated at 25° for 3 days along with a control lacking the alkylating agent.

Periodically 50  $\mu$ l aliquots of these solutions were assayed for residual enzyme activity by their addition to 2 ml solutions containing 50  $\mu$ M Boc-Tyr-ONp (0.05 M citrate, 1.0 M NaCl, pH 6.5) and following the increase in absorption of each at 347.5 nm.

A 1 ml solution of subtilisin BPN' (0.10 mg/ml, 5.0 mM  $K_2HPO_4$ , 1.0 M NaCl, pH 7.5) was treated with the alkylating agent in exactly the same manner as chymotrypsin  $A_\alpha$  to give a final enzyme concentration of 95  $\mu$ g/ml. The assay buffer contained 0.01 M  $K_2HPO_4$  instead of citrate.

pH Dependence of the Inactivation of Thermolysin. The buffers used were: Pipes (pH 6.00) Hepes (pH 6.50-7./0), and Tris (pH 8.00-8.95); all were 0.10 M and contained 0.01 M  $CaCl_2$ .

Thermolysin (4.2  $\mu$ M) was incubated at 25° in the appropriate buffer with  $ClCH_2CO-DL-(N-OH)Leu-OCH_3$  (2.3 mM) containing 5% DMF. Periodically 50  $\mu$ l aliquots were taken and assayed for residual enzyme activity.

Active Site Protection of Thermolysin. Thermolysin (4.4  $\mu$ M, Tris buffer, pH 7.2, 5% DMF) was incubated at 25° with  $ClCH_2CO-DL-(N-OH)Leu-OCH_3$  (4.5 mM) in the presence and absence of the inhibitor Z-Phe-OH (1.7 mM) with a reported  $K_I$  of 0.51 mM. This inhibitor binds at the active site (Kester and Matthews, 1977). Periodically aliquots were assayed for residual enzyme activity.

Thermolysin (4.2  $\mu$ M, Tris buffer, pH 7.2) was also incubated at 25° with  $ClCH_2CO-DL-(N-OH)Leu-OCH_3$  (0.50 mM, 5% DMF) for 1.5 hr in the presence and absence of P-Leu-Trp-OK (49 M). These solutions, along with a control containing the enzyme and competitive inhibitor only, were dialyzed 22 hr against 3 changes of buffer, then assayed for enzyme activity.

Incorporation of N-(1-<sup>14</sup>C) Chloroacetyl-DL-N-hydroxyleucine methyl ester into Thermolysin. A 15 ml solution of purified thermolysin (20 mg) was treated with a 1 ml solution of (<sup>14</sup>C) ClCH<sub>2</sub>CO-(N-OH)Leu-OCH<sub>3</sub> (10 mg/ml) containing 10% DMF. The pH of the enzyme-inhibitor solution was then adjusted to 7.5 with 0.2 M NaOH. This solution was incubated 1 hr at room temperature; however, complete loss of enzymatic activity toward FA-Gly-Leu-NH<sub>2</sub> was achieved after only 10 min.

Inactivated thermolysin was separated from the excess alkylating agent by passage through a 1 x 25 cm column of Sephadex G-25. The concentrations of the protein fractions were determined by measuring the absorbance of each at 280 nm. The fractions containing the excess alkylating agent were located by the characteristic red color produced by the addition of 1 drop of a 10% solution of methanolic ferric chloride to the fractions following the elution of the protein. The radioactivity of the 4 peptide containing fractions was measured.

A 2 ml sample of a 7.72 μM solution of (<sup>14</sup>C) thermolysin was dialyzed against 1.0 mM NaOH for 10 hr at 25°. Periodically the radioactivity in the dialysis tubing was measured on 100 μl aliquots. A 2.5 ml sample from the same stock (<sup>14</sup>C) thermolysin solution was dialyzed against a 1.0 M hydroxylamine solution (0.1 M Tris, 1.0 M NaCl, 0.01 M NaCl<sub>2</sub>, pH 9.0) for 10 days at room temperature. The radioactivity inside the dialysis tubing was measured as before. The possibility of protein leakage from the dialysis tubing was checked by measuring the absorbance at 280 nm of the solution inside the tubing at the termination of dialysis.

Cyanogan Bromide Cleavage of (<sup>14</sup>C) Thermolysin was performed as described by Titani et al. (1972a). The lyophilized cleavage peptides

were dissolved in 1 ml of 1.0 M formic acid containing 8 M urea and chromatographed on a 1 x 50 cm column of Bio-Gel P-100 equilibrated and eluted with 1.0 M formic acid. Fractions of 1.8 ml were collected at a flow rate of 12 ml per hr. An aliquot (200  $\mu$ l) from each fraction was added to 800  $\mu$ l of water and the radioactivity present measured. The individual fragment peptides were purified by the method of Titani et al. (1972a).

Thin Layer Chromatography of Clostridiopeptidase A Cleavage of N-Carbobenzyloxy-glycyl-L-prolyl-L-leucyl-glycolyl-L-prolinamide. To a 1.0 ml solution of Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> (1.0 mM, 0.10 M Tris, 0.01 M CaCl<sub>2</sub>, pH 7.2) containing 1% methanol was added 100  $\mu$ l of a solution of clostridiopeptidase A (0.01 M calcium acetate) to give a final enzyme concentration of 53  $\mu$ g/ml. Periodically, tlc analysis was performed on 2  $\mu$ l aliquots by developing on silica gel-G with chloroform-methanol (5:1, v/v). The above analysis was performed at pH 8.8, with the same enzyme solution after boiling 1 min, and with the substrate solution containing 1,10-phenanthroline (10 mM).

pH-Stat Assay of Clostridiopeptidase A Activity was performed on a Radiometer instrument. A typical assay was performed at pH 7.5. To a 1.0 ml aqueous solution of an appropriate concentration of Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> containing either 2% DMF or 2% methanol was added 50  $\mu$ l of a clostridiopeptidase A solution (0.01 M calcium acetate). The concentration of the titrating NaOH was around 0.05 M for all assays. The addition of NaOH to maintain pH 7.5 in the substrate solution was followed with time after the addition of enzyme. Background hydrolysis of the substrate was measured in the absence of enzyme.

Determination of  $K_m$  for Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> and Clostridiopeptidase A. The concentrations of Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> ranged from 5.0-0.55 mM. The assay concentration of the enzyme was 54 µg/ml or about 2 µM using a molecular weight of 26,000 for the enzyme (Worthington Enzyme Manual, 1972).

Dependence of the concentration of Clostridiopeptidase A on the hydrolysis of Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub>. The concentration of clostridiopeptidase A ranged from 54-10.8 µg/ml in the assay.

The pH Dependence of  $K_m$  and  $k_{cat}$  on the Hydrolysis of Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> by Clostridiopeptidase A. The determination of the  $K_m$  and  $k_{cat}$  values over the pH range of 6-8.5 was performed with the substrate concentrations ranging from 3-0.75 mM and containing 2% methanol. The concentration of enzyme was 2.7 µM. The background hydrolysis of substrate in the absence of enzyme was measured for each pH value studied.

pH-stat Assay of Clostridiopeptidase A in the Presence of 1,10-Phenanthroline and  $\alpha$ -Toluenesulfonyl Fluoride. The concentration of Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> was 10.2 mM and contained 2% DMF and 1,10-phenanthroline (8.6 mM) or  $\alpha$ -toluenesulfonyl fluoride (10 mM). The assay concentration of clostridiopeptidase A was 71 µg/ml.

Inhibition Studies of Clostridiopeptidase A with Hydroxamic acids, Phosphoramidates, Thiol Peptides, and Substrate Hydrolysis Products. To 1 ml of substrate solution of the appropriate concentration (4 or 1 mM) was added 100 µl of the appropriate inhibitor solution (10 - 1 mM). The pH was adjusted to 7.5 and 50 µl of clostridiopeptidase A solution (0.01 M calcium acetate) was added to give a final enzyme concentration of 60 µg/ml. The  $K_I$  values were determined by Dixon plots.

Hydrolysis of Z-Gly-Pro-Leu-Gly-Pro-NH<sub>2</sub> by Clostridiopeptidase A.

The composition of the reaction mixture was as follows: 0.01 M calcium acetate, pH 7.5, 4.1  $\mu$ M clostridiopeptidase A, and the appropriate substrate concentration (7 - 0.9 mM) were placed in a test tube to give a total volume of the reaction solution of 1.2 ml containing 4% DMF. The assay was carried out at 25° and the reaction was initiated by the addition of 100  $\mu$ l of enzyme solution to the substrate solution. At appropriate time intervals, 250  $\mu$ l of reaction solution were pipetted out into a test tube containing 250  $\mu$ l of 0.1 M HCl to stop the reaction. The amount of  $\alpha$ -amino group released was determined colorimetrically at 570 nm according to the ninhydrin method of Rosen (1957). Glycylprolinamide was used as the ninhydrin color standard:  $\epsilon_{570}^M = 1.05 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . The hydrolysis was also followed using thin layer chromatography, n-butanol-acetic acid-water (4:1:1, v/v) was the solvent system used.

## CHAPTER III

## RESULTS

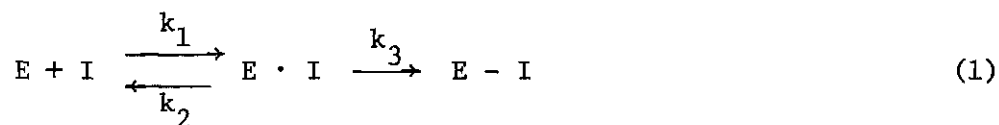
Haloacetyl Amino Acid Esters. Earlier studies with carboxypeptidase A (Hass and Neurath, 1971a, b) and carboxypeptidase B (Plummer, 1971) suggested a possible route to the active site directed inhibition of thermolysin. The primary specificity for substrate binding for each of these metalloproteases is determined by the  $S_1'$  subsite. Thermolysin however does not like a terminal carboxyl group. Therefore the compounds  $\text{BrCH}_2\text{CO-Phe-OCH}_3$  and  $\text{BrCH}_2\text{CO-L-MeLeu-OCH}_3$ , containing a favorable  $P_1'$  residue and a blocked carboxyl group, were synthesized as potential thermolysin inhibitors. However neither of these compounds measurably inhibited thermolysin after 48 hr incubation. The absence of irreversible inactivation was probably the result of poor binding to the active site of thermolysin since both compounds showed no inhibition of the hydrolysis of  $\text{FA-Gly-L-Leu-NH}_2$ . We then decided to extend the peptide chain of the inhibitor and synthesized  $\text{BrCH}_2\text{CO-L-MeLeu-L-Ala-OCH}_3$ . Earlier studies with serine proteases and chloromethyl ketones have demonstrated that inhibitors with extended peptide chains were more effective and in the case of elastase only longer peptide inhibitors would react with the enzyme (Powers, 1977). This approach, however, failed with thermolysin since no noticeable inhibition was observed with the bromoacetyl dipeptide.

N-Chloroacetyl-DL-N-hydroxyleucine methyl ester. We next approached the problem by searching for good reversible inhibitors of thermolysin to which would be attached an alkylating functional group. At about this

time peptide hydroxamic acid derivatives were demonstrated to be potent inhibitors of thermolysin with  $K_I$  values in the  $\mu\text{M}$  range (Nishino and Powers, 1978). Since the hydroxamic acid moiety of the inhibitors was apparently responsible for the increased binding to the enzyme, we investigated other N-hydroxy compounds. The compound DL-2-hydroxyamino-4-methylpentanonitrile was a competitive inhibitor of thermolysin ( $K_I = 0.10 \text{ mM}$ ). This led to the synthesis of DL-2-(N-bromoacetyl-N-hydroxyamino)-4-methylpentanonitrile which was found to rapidly and irreversibly inactivate thermolysin at pH 7.2 (0.10 M Tris, 0.01 M  $\text{CaCl}_2$ ) containing 5% DMF at  $25^\circ$ .

Extensive use of the nitrile as an affinity label for thermolysin was complicated by its being an oil which was difficult to purify. As a result of this undesirable physical property, a search was made for a crystalline compound which possessed the N-haloacetyl-N-hydroxy configuration of the nitrile. The required molecule ( $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$ ) had been synthesized by Cook and Slater (1956) but for an entirely different purpose. This compound was almost identical in reactivity toward thermolysin as the nitrile.

Kinetics of Inactivation. The active site directed irreversible inhibition of an enzyme may be represented by Eq. 1 where the formation of a reversible enzyme-inhibitor complex occurs prior to the inactivation reaction (Kitz and Wilson, 1970). The observed rate constant



of inactivation is a function of inhibitor concentration and can be expressed in the convenient reciprocal form given by Eq. 2. By using inhibitor concentrations

$$\frac{1}{k_{\text{obs}}} = \frac{K_I}{k_3 I} + \frac{1}{k_3} \quad (2)$$

in the vicinity of  $K_I$  (the dissociation constant for the reversible enzyme-inhibitor complex) it is often possible to determine  $K_I$  and  $k_3$  (the limiting rate of inactivation) by plotting  $1/k_{\text{obs}}$  vs  $1/I$ . A plot of  $1/k_{\text{obs}}$  vs  $1/I$  for the irreversible inactivation of thermolysin at pH 7.2 by  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  using the least squares method gave  $K_I = 7.5 \pm 2.2$  mM and  $k_3 = 7.5 \pm 2.0 \times 10^{-3}$  sec<sup>-1</sup> ( $I = 10.5 - 2.1$  mM). A similar plot for thermolysin and 2-(N-bromoacetyl-N-hydroxyamino)-4-methylpentanonitrile gave  $K_I = 0.80 \pm 0.36$  mM and  $k_3 = 7.4 \pm 3.3 \times 10^{-3}$  sec<sup>-1</sup> ( $I = 0.87 - 0.12$  mM). The limiting rates of inactivation ( $k_3$ ) were essentially identical for the two compounds, but the bromoacetyl-nitrile was bound 10 fold more tightly by the enzyme.

#### Treatment of Thermolysin with $\alpha$ -Bromoisocaproylhydroxamic acid.

If  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  was binding to the active site of thermolysin, with the isobutyl group occupying the  $S_1'$  subsite and coordination of the hydroxamic acid to the zinc, then the chloroacetyl group would spend most of the time closer to the active site Glu-143 rather than the His-231 residue. This proximity effect would tend to promote alkylation of the Glu-143 carboxylate over the imidazole of His-231. In an attempt to distinguish between these two possible sites of alkylation, thermolysin was treated with  $\alpha$ -bromoisocaproylhydroxamic acid. Assuming again that

the isobutyl group would occupy the  $S_1'$  subsite, with coordination of the hydroxamic acid group to the active site zinc atom, the  $\alpha$ -bromo group would be considerably closer to the His-231 residue than to the carboxylate group of Glu-143, promoting alkylation of the imidazole. However, the half time of inactivation of thermolysin by  $\alpha$ -bromoisocaproylhydroxamic was 58 hr, 3 orders of magnitude longer than for the chloroacetyl compound. This result was the first indication that the primary site of alkylation may not be the His-231 residue but possibly Glu-143.

Treatment of Thermolysin with N-(3-methyl-1-methoxycarbonylbutyl)-3-iso-butyloxaziridine and N-Benzylidene-DL-leucine-N-oxide methyl ester.

These two compounds were synthesized as potential suicide substrates of thermolysin. However, thermolysin showed no loss of activity after 3 days incubation. These compounds showed no binding to thermolysin, indicating that the functionality present had no opportunity to alkylate a susceptible active site residue of the enzyme.

pH Dependence of Inactivation. The pH dependence of the irreversible inactivation of thermolysin by  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  was bell shaped (Fig. 1) and similar to the pH profile of catalysis reported by Pangburn and Walsh (1975). The pH optimum for alkylation was around 7.5. The inflection points around pH 7.0 and 7.7 indicate at least two different ionizable groups involved in the inactivation of thermolysin. Two such groups could possibly be the active site Glu-143 and His-231 (Pangburn and Walsh, 1975). However, the pH profile for inactivation of thermolysin is complicated by the possible ionization of the N-OH oxygen of the inhibitor. Since the inhibitor concentration was of the same order of magnitude as the  $K_I$  value, the pH dependence of the limiting

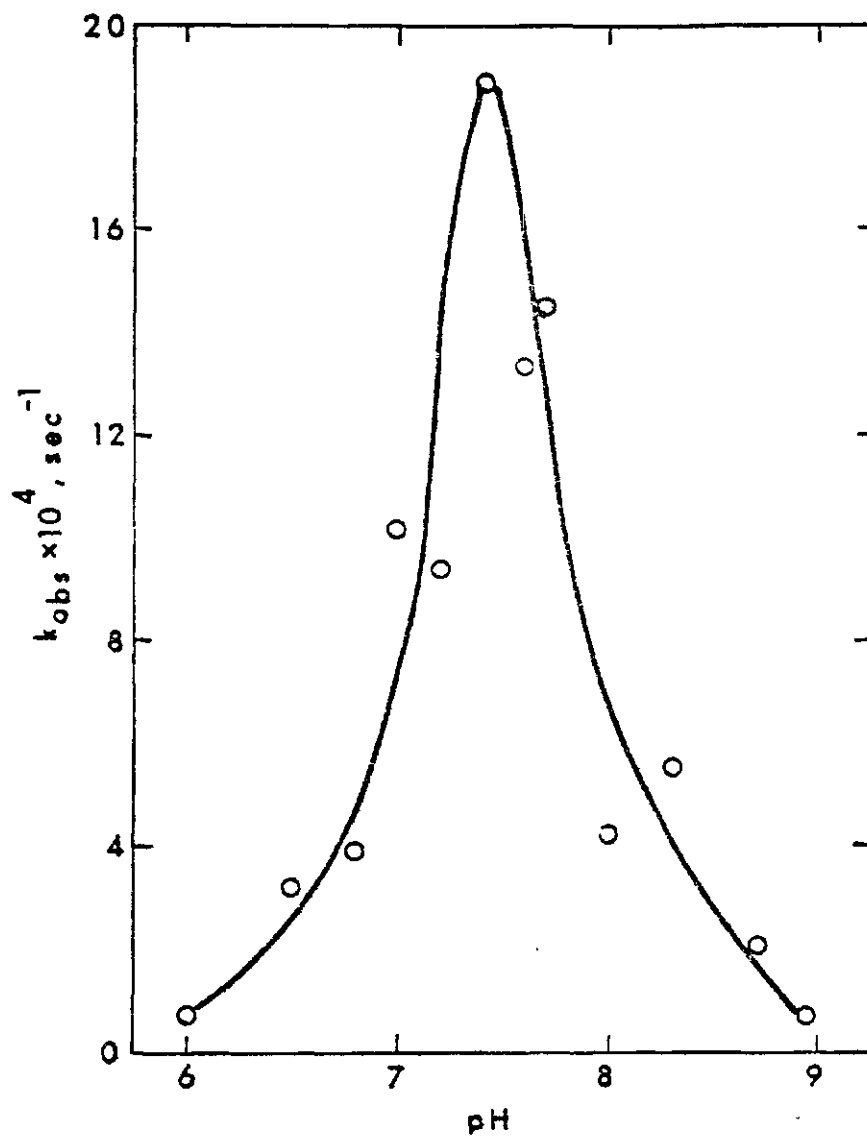


Figure 1. pH dependence of the inactivation of thermolysin ( $4.2 \mu\text{M}$ ) by  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  ( $2.3 \text{ mM}$ ). The buffers used are described in the text.

rate of inactivation ( $k_3$ ) cannot be distinguished from the pH dependence of binding ( $K_I$ ). As a result, Fig. 1 represents the pH dependence of both  $k_3$  and  $K_I$ , and is shown only to illustrate the practical pH range for alkylation.

Active Site Protection of Thermolysin. The inactivation of thermolysin by  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  in the presence and absence of the competitive inhibitor Z-Phe-OH ( $K_I = 0.51 \text{ mM}$ ) is shown in Fig. 2. The presence of the competitive inhibitor resulted in a reduction in the rate of inactivation of the enzyme. This inhibitor has been shown by X-ray crystallography to bind at the active site (Kester and Matthews, 1977). The  $k_{\text{obsd}}$  in the presence of inhibitor was  $1.0 \times 10^{-3} \text{ s}^{-1}$ . The value calculated from the equation  $k_{\text{obsd}} = k_3 I / (K_I(1+I'/K_I') + I)$  where  $I$  is the irreversible inhibitor and  $I'$  is Z-Phe-OH, was  $0.90 \times 10^{-3} \text{ s}^{-1}$ .

When thermolysin was treated with as little as a 12 fold molar excess of the competitive inhibitor P-Leu-Trp-OK ( $K_I \sim 0.1 \text{ }\mu\text{M}$ ) (Komiyama *et al.*, 1975; Kam and Powers, unpublished results) immediately prior to incubating 1.5 hr in the presence of  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$ , there was no detectable loss of enzyme activity due to irreversible inactivation as compared with a control following dialysis. A control lacking the competitive inhibitor showed complete loss of enzyme activity after 20 min incubation.

Specificity of the Inhibition. A comparison of the rates of inactivation of 3 metalloendoproteases by  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  (8.4 mM) was made in 0.10 M Tris, 0.1 M  $\text{CaCl}_2$ , pH 7.2 buffer containing 5% DMF at 25°. The  $k_{\text{obs}}/I$  values of 400, 5.4 and  $6.4 \text{ M}^{-1} \text{ s}^{-1}$  were observed respectively for thermolysin, neutral protease A and neutral protease B.

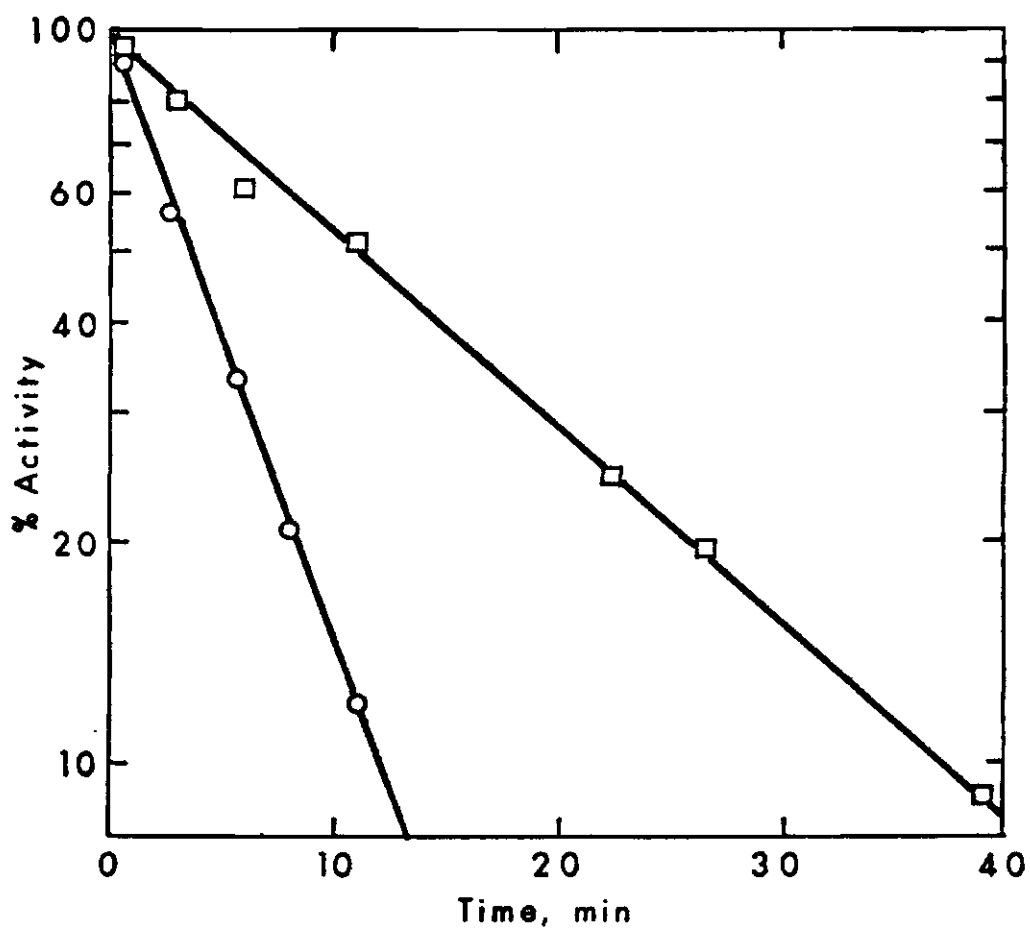


Figure 2. Active site protection against inactivation. Thermolysin (4.4  $\mu\text{M}$ ) in 0.10 M Tris, 0.01 M  $\text{CaCl}_2$ , pH 7.2 containing 5% DMF was incubated with  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  (4.5 mM) in the presence (■) and absence (○) of the competitive inhibitor Z-Phe-OH (1.7 mM). The enzyme was assayed for residual activity as described in the text.

Neutral proteases A and B from B. subtilis were irreversibly inhibited nearly 2 orders of magnitude slower than thermolysin. Neutral protease B hydrolyzed FA-Gly-Leu-NH<sub>2</sub> 3 times faster than neutral protease A, but both these enzymes were alkylated at about the same rate.

Carboxypeptidase A was irreversibly inhibited by ClCH<sub>2</sub>CO-DL-(N-OH)-Leu-OCH<sub>3</sub> with a half time of inactivation of more than 3 days. Under the same conditions carboxypeptidase A was not measurably inactivated by 2-(N-bromoacetyl-N-hydroxyamino)-4-methylpentanonitrile.

Chymotrypsin A<sub>α</sub> and subtilisin BPN' showed no measurable loss of their enzymatic activities after 3 days of incubation in the presence of ClCH<sub>2</sub>CO-(N-OH)Leu-OCH<sub>3</sub> when compared to their controls.

Incorporation of N-(1-<sup>14</sup>C) Chloroacetyl-DL-N-hydroxy-leucine Methyl Ester into Thermolysin. The incorporation of carbon 14 labeled ClCH<sub>2</sub>CO-DL-(N-OH)Leu-OCH<sub>3</sub> into thermolysin resulted in  $0.97 \pm 0.03$  inhibitor molecules attached to 1 enzyme molecule. The 1 to 1 stoichiometry was not affected by extended incubation of the enzyme for up to 2 days in the presence of the inhibitor following the complete loss of enzymatic activity. Carbon 14 labeled thermolysin lost 99% of the incorporated radioactivity after treatment with 1.0 mM NaOH for 8 hr. There was no significant recovery of enzymatic activity under these conditions. None was expected since a control thermolysin solution lost 99% of its activity after identical treatment. However, on treatment with 1.0 M hydroxylamine at pH 9.0 in the presence of 0.01 M CaCl<sub>2</sub> the release of radioactivity was quite slow ( $t_{1/2} = 175$  hr). There was no recovery of thermolysin's ability to hydrolyze FA-Gly-L-Leu-NH<sub>2</sub>; whereas, a control with nonalkylated thermolysin retained 18% enzymatic activity after 216 hr in

the presence of 1.0 M hydroxylamine and pH 9.0.

Amino Acid Analysis of Alkylated Thermolysin. Thermolysin was alkylated with  $\text{ClCH}_2\text{CO}-(\text{N-OH})\text{Leu-OCH}_3$  and an amino acid analysis was performed and compared with that for unlabeled enzyme (available in the supplementary material). The analysis was not corrected for the decomposition of labile amino acids nor for incomplete hydrolysis of the bulky residues. The results showed that under the same conditions of hydrolysis there was no difference between the amino acid analyses of alkylated and nonalkylated thermolysin. Carboxymethyl histidine and carboxymethyl tyrosine did not appear in the amino acid analysis of alkylated thermolysin.

Cyanogen Bromide Cleavage of Thermolysin Alkylated with N-(1<sup>14</sup>C) Chloroacetyl-DL-N-hydroxyleucine Methyl Ester. The chromatography pattern of the cyanogen bromide cleavage products of alkylated thermolysin on Bio-Gel P-100 is shown in Fig. 3. The radioactivity was concentrated in the first and third peaks. These two peaks contain both the  $F_I$  (residues 121-205) and  $F_{III}$  (residues 1-120) peptides and none of the  $F_{II}$  (residues 206-316) fragment (Titani *et al.*, 1972a). Fragment  $F_I$  was purified from the third peak by the method of Titani *et al.*, (1972a) which involves removal of  $F_{III}$  by precipitation. The resultant  $F_I$  showed a single band on gel electrophoresis ( $F_{III}$  is clearly distinguishable). The amino acid analysis of the pure  $F_I$  is given in the supplementary material. The number of inhibitor molecules attached to the  $F_I$  fragment was determined by measuring the radioactivity associated with 7.4 nmol of purified peptide. The amount of  $F_I$  peptide used was determined by amino acid analysis based on the number of Leu present and the known content of this residue (Titani *et al.*, 1972a). Each molecule of  $F_I$  peptide was found to have 0.96

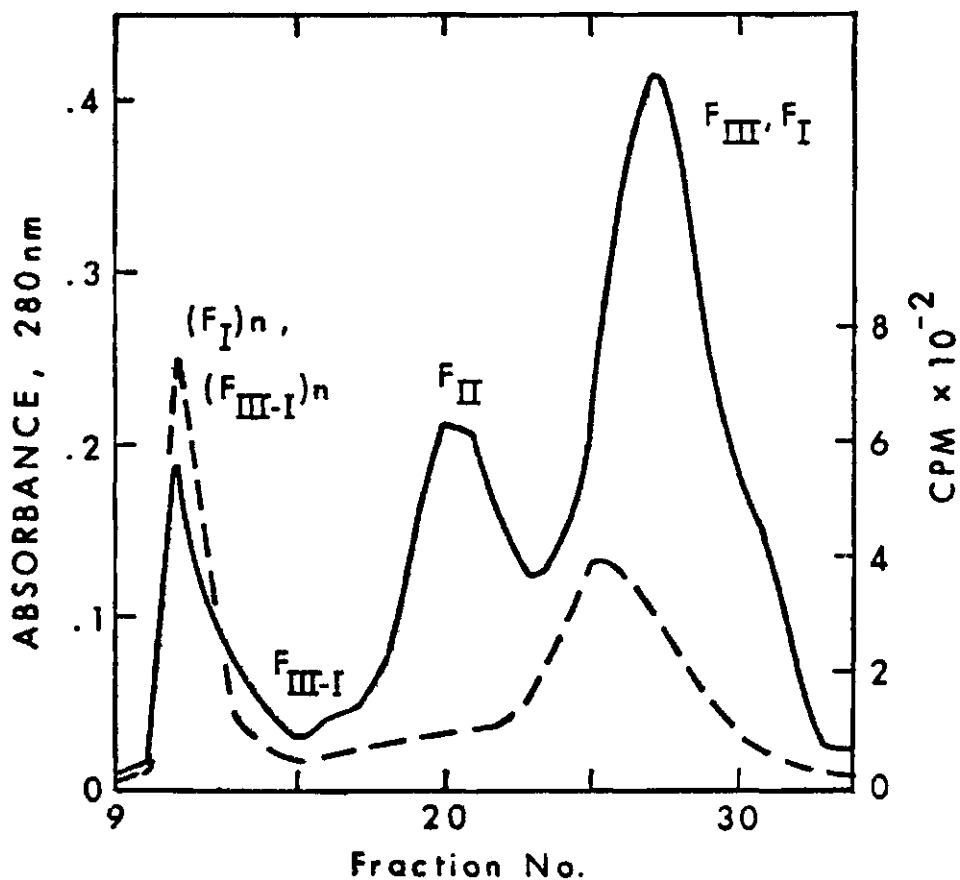


Figure 3. Separation of the cyanogen bromide fragments of thermolysin on a 1 x 50 cm column of Bio-Gel P-100 equilibrated and eluted with 1.0 M formic acid. Fractions (1.8 ml) were monitored both at 280 nm (solid line) and for radioactivity (broken line). Peaks were labeled as described by Titani *et al.* (1972a).

inhibitor molecules attached.

Ester Cleavage of Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> by Clostridiopeptidase A. The thin layer chromatography experiments indicated that the spot with  $R_f^3$  value of 0.65 (Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub>) diminished with time while a new spot ( $R_f^3$  0.26) identical with glycolylprolinamide increased with time following the addition of clostridiopeptidase A at pH 7.2 and 25°. A control lacking enzyme showed only a single spot ( $R_f^3$  0.65) after 2 days incubation. The presence of 1,10-phenanthroline (10 mM) eliminated clostridiopeptidase A cleavage of Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> as determined by tlc. Tris buffer at pH 8.8 in the absence of enzyme resulted in the same tlc cleavage pattern as in the presence of enzyme. Boiling the enzyme solution also eliminated ester cleavage.

pH-Stat Assay of Clostridiopeptidase A Esterase Activity. The pH-stat esterase activity of clostridiopeptidase A using Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> was conveniently performed using either 2% DMF or 2% methanol. However, 5% DMF noticeably inhibited the enzyme, whereas, 5% methanol only resulted in a slight loss of enzymatic activity. A convenient enzyme concentration was around 50 µg/ml in the assay. The average background rate of addition of 0.05 M NaOH was  $2.0 \times 10^{-10}$  moles sec<sup>-1</sup>. The Km for the substrate Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> at pH 7.5 was determined to be  $1.3 \pm 0.1$  mM. This compares with a Km of 1.5 mM for Z-Gly-Pro-Leu-Gly-Pro-Gly-OCH<sub>3</sub> (Solov'eva *et al.*, 1970) at pH 7.5. The concentration dependence of clostridiopeptidase A on the hydrolysis of Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> is shown in Fig. 4. The hydrolysis of ester is linearly dependent on enzyme concentration. The assay is usable easily down to 10 µg/ml of enzyme in the assay solution.

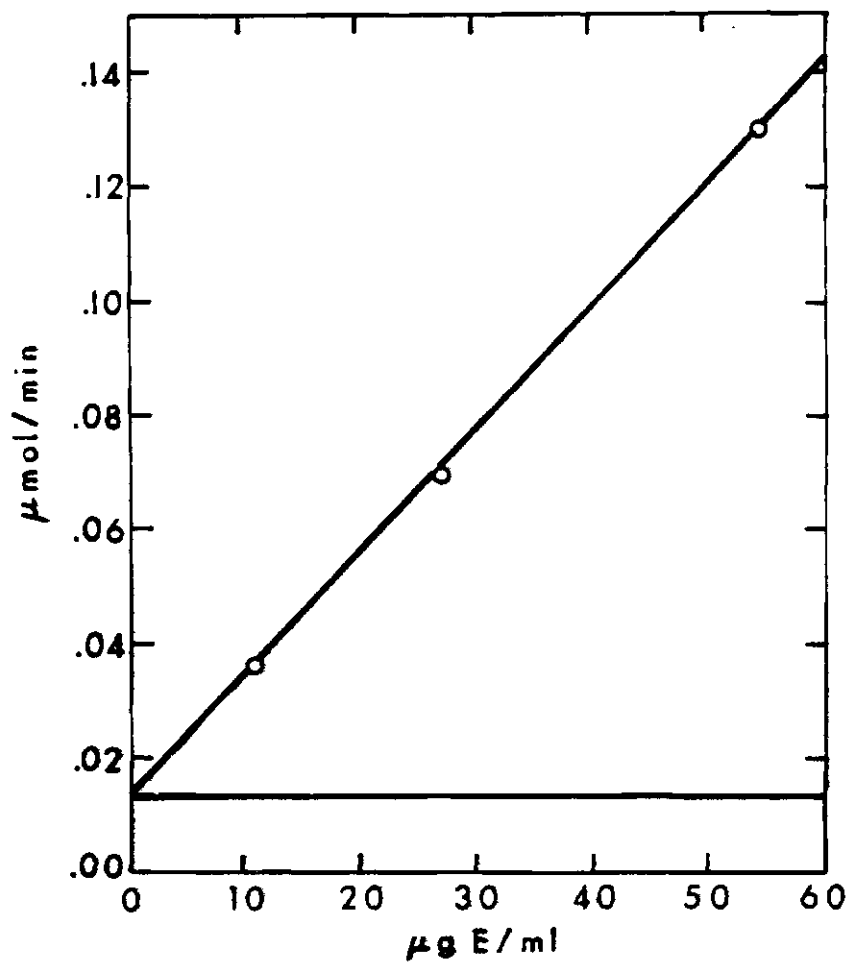


Figure 4. Dependence of the concentration of clostridiopeptidase A on the hydrolysis of Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub>. The concentration of substrate was 10.2 mM and contained 2% DMF. The assay pH was 7.5.

The pH Dependence of  $K_m$  and  $k_{cat}$  for the Esterase Activity of Clostridiopeptidase A is shown in Fig. 5 and is consistent with that reported by Yagisawa et al. (1965) for the hydrolysis of Z-Gly-Pro-Leu-Gly-Pro-OH. The pH range (6-8.5) studied resulted in a negligible decrease in hydrolysis rate due to denaturation (Yagisawa et al., 1965). The value of  $k_{cat}$  was nearly independent of pH over the range chosen, whereas,  $K_m$  decreased as the pH increased. Since the substrate is neutral, the decrease of  $K_m$ -values might be due to a dissociable group of the enzyme molecule.

Treatment of Clostridiopeptidase A with 1,10-Phenanthroline and  $\alpha$ -Toluenesulfonyl Fluoride. As in the case of the tlc studies, 1,10-phenanthroline in the substrate solution inhibited the ester hydrolysis of Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> by clostridiopeptidase A by reducing the pH-stat rate to background levels. However, after treatment of the enzyme with  $\alpha$ -toluenesulfonyl fluoride, there was no loss of enzymatic activity after incubating 20 min at pH 7.5. It is unlikely, therefore, that the ester hydrolysis was due to the presence of contaminating serine proteases.

Inhibition Studies of Clostridiopeptidase A Using the pH-Stat. The inhibition results are shown in Table 1. All of the compounds which inhibited the enzyme were competitive inhibitors. Consistent with the indications that clostridiopeptidase A is a zinc containing metalloendroprotease, the best inhibitor was the chelating tripeptide Z-Gly-Pro-Leu-NHOH. This peptide was shown not to be cleaved by clostridiopeptidase A using tlc methods. As expected, Z-Gly-Gly-Leu-NHOH was a poorer inhibitor due to the wellknown preference for a prolyl residue at the P<sub>2</sub> site by

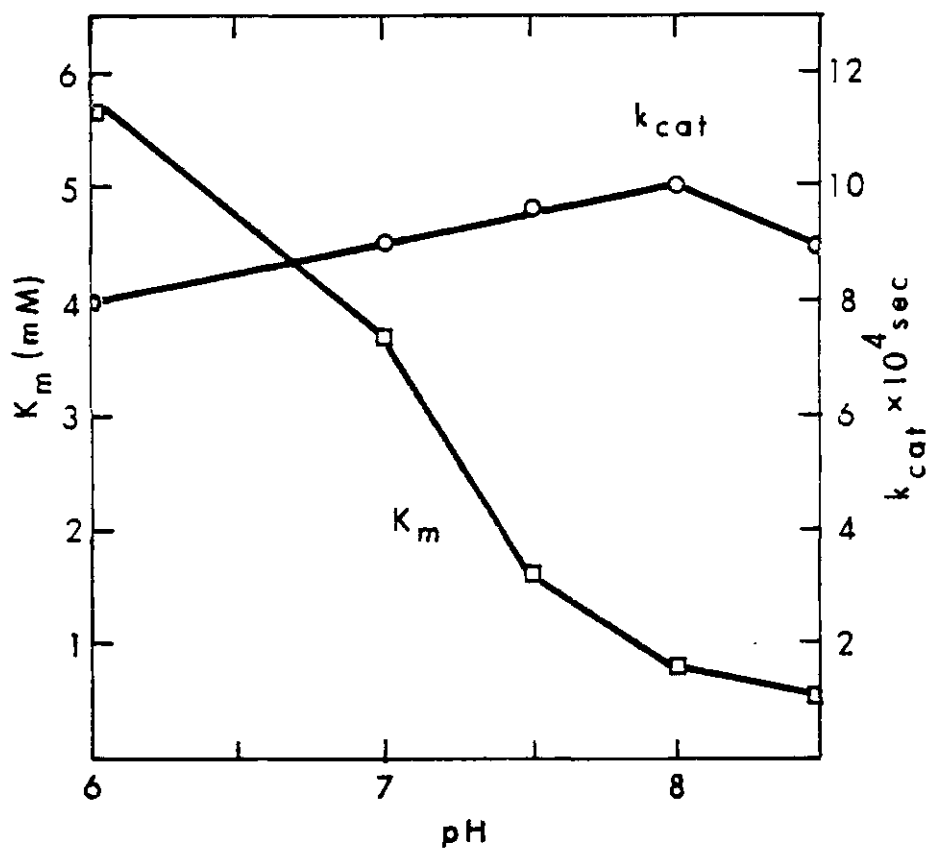


Figure 5. The pH dependence of  $K_m$  ( $\square$ ) and  $k_{cat}$  (O) on the esterase activity of clostridiopeptidase A. The substrate concentrations ranged from 3 - 0.75 mM and contained 2% methanol. The enzyme concentration was 2.7  $\mu$ M.

Table 1. Inhibition of Clostridiopeptidase A<sup>a</sup>

Compound	Mode	K <sub>I</sub> (mM)
Glycolylprolimamide	Competitive	15
Z-Gly-Pro-Leu-NHOH	Competitive	0.2
H-Gly-Pro-Leu-(N-HO)Gly-Pro-OH <sup>b</sup>	None	--
Boc-Gly-Pro-Leu-(N-OH)Gly-Pro-OH	Competitive	17
HONHCOCH <sub>2</sub> CO-Pro-NH <sub>2</sub>	Competitive	4.6
Boc-Gly-Pro-Leu-(N-OH)Gly-OEt <sup>c</sup>	None	--
Z-Gly-Pro-Leu-OH	Competitive	4.3 <sup>d</sup>
Z-Gly-Gly-Leu-NHOH	Competitive	18.9
Z-Gly-Leu-NHOH	Competitive	1.5
HSCH <sub>2</sub> CH(B <sub>Z</sub> )CO-Ala-Gly-NH <sub>2</sub>	Competitive	4.4
P-Ile-Ala-OK <sup>b</sup>	None	--
HONHCOCH(i-Bu)CO-Ala-Gly-NH <sub>2</sub>	Competitive	4.1

- a. Concentrations of Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub> were 4 and 1mM and contained 2% methanol, pH was 7.5, the concentration of clostridiopeptidase A was 60 µg/ml. K<sub>I</sub> values were determined by Dixon plots.
- b. No inhibition at a maximum inhibitor concentration of 3 mM.
- c. No inhibition at a maximum inhibitor concentration of 1 mM due to solubility restrictions.
- d. Yagisawa et al. (1965) report a K<sub>I</sub> of 4.9 mM.

the enzyme. Both products of the ester hydrolysis were reasonably good inhibitors of clostridiopeptidase A; with the N-terminal fragment being the better inhibitor, with a  $K_I$  comparing favorably with that reported by Yagisawa et al. (1965). The good thermolysin inhibitor P-Ile-Ala-OK showed no binding to clostridiopeptidase A, most likely due to the complete absence of prolyl residues and the unfavorable placement of an alanyl residue at the  $P_1'$  site. Significantly the N-substituted hydroxamic acid peptides showed no or weak inhibition of clostridiopeptidase A. Finally, the alkylating compounds  $\text{CH}_3\text{CO-Ala-Ala-Pro-IleCH}_2\text{Cl}$  (1 mM) and  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  (1 mM) did not inhibit clostridiopeptidase A after 3 days incubation.

The Hydrolysis of Z-Gly-Pro-Leu-Gly-Pro-NH<sub>2</sub> by Clostridiopeptidase

A followed by the ninhydrin method resulted in a  $K_m$  of  $4.0 \pm 1.5$  mM. The  $k_{cat}$  was  $2.9 \pm 1.1 \times 10^{-3} \text{ sec}^{-1}$  or about 3 times that for the ester which was  $0.89 \pm 0.02 \times 10^{-3} \text{ sec}^{-1}$ . The  $k_{cat}/K_m$  values were  $0.73 \pm 0.11$  and  $0.68 \pm 0.02 \text{ M}^{-1} \text{ sec}^{-1}$  respectively at pH 7.5. A smaller  $k_{cat}$  value for the ester in comparison with the peptide is consistent with the results for the metalloendoprotease thermolysin and the neutral proteases A and B from B. subtilis.

## CHAPTER IV

## DISCUSSION

The molecular structures of carboxypeptidase A (Hartsuck and Lipscomb, 1971), carboxypeptidase B (Schmid and Herriott, 1976), and Thermolysin (Matthews *et al.*, 1972b) have been determined to atomic resolution by X-ray crystallography. The active sites of these three metalloproteases have many features in common. They include the zinc atom which polarizes the scissile peptide bond of a substrate, a glutamic acid residue which catalyzes the addition of water to that peptide bond and a group which donates a proton to the scissile peptide bond (His in thermolysin and probably Tyr in carboxypeptidase). Although detailed structural information is not yet available for other important metalloproteases such as collagenase or the angiotensin converting enzyme, analogous catalytic groups would be expected to be involved in their active sites. The individual members of the metalloprotease family differ of course in their specificity for peptides or peptide-like structures.

It would be valuable to have available a class of affinity labels for metalloproteases which would be both reactive and specific. The inhibitors might incorporate a reactive group directed toward one of the catalytic groups of a metalloprotease into the structure of a good peptide-like substrate for a particular metalloprotease. Specificity toward individual metalloproteases could probably then be achieved by matching the

structure of the inhibitor to that of a good substrate of a particular metalloprotease. Peptide chloromethyl ketones are one example of a class of inhibitors where considerable specificity for individual members of the serine protease family can be achieved by alterations in the amino acid sequence of the peptide portion of the inhibitor (for a review, see Powers, 1977). This research was begun as a first step toward the development of such a class of inhibitors for metalloproteases.

The carboxypeptidase A and B affinity labels which had been previously described (Hass and Neurath, 1971a and b; Hass et al., 1972) led us to synthesize similar compounds in an attempt to extend these inhibitors to the metalloendoprotease thermolysin. However, these compounds ( $\text{BrCH}_2\text{CO-Phe-OCH}_3$ ,  $\text{BrCH}_2\text{CO-L-MeLeu-OCH}_3$ , and  $\text{BrCH}_2\text{CO-L-MeLeu-Ala-OCH}_3$ ) showed no indication of being thermolysin inhibitors. It is difficult to explain why the first compound ( $\text{BrCH}_2\text{CO-Phe-OCH}_3$ ) was not an inhibitor since Gly-Phe-NH<sub>2</sub> binds to thermolysin, although the  $K_I$  value is high (53 mM) (Feder et al., 1974). This compound may be a substrate of thermolysin with a large  $K_m$ , the result being that any competitive binding relative to the assay substrate would not be observed since solubility restrictions limited the concentration of the alkylating agent which could be utilized. The lack of inhibition by  $\text{BrCH}_2\text{CO-L-MeLeu-OCH}_3$  is more easily explained in terms of poor binding since the N-methyl group could result in severe steric problems at the active site of the enzyme. In addition, the replacement of the P<sub>1</sub>' amide hydrogen with a methyl group eliminates the hydrogen bond with the carbonyl oxygen of Ala 113 of thermolysin (Weaver et al., 1977). Even extension of the chain of the inhibitor to  $\text{BrCH}_2\text{CO-L-MeLeu-L-Ala-OCH}_3$  had no effect on the inhibition.

Replacement of the P<sub>1</sub>' amide hydrogen with an hydroxyl group had a profound effect on the inhibitory properties of the haloacetyl amino acid analogs. Both DL-2-(N-bromoacetyl-N-hydroxyamino)-4-methylpentanonitrile and ClCH<sub>2</sub>CO-DL-(N-OH)Leu-OCH<sub>3</sub> bound to and rapidly irreversibly inactivated thermolysin. The increased binding of the N-hydroxypeptides to thermolysin is probably due to coordinating of the hydroxamic acid functional group to the active site zinc atom of thermolysin. The K<sub>I</sub> of the bromoacetyl nitrile is 0.80 mM and that of ClCH<sub>2</sub>CO-DL-(N-OH)Leu-OCH<sub>3</sub> is 7.5 mM while most dipeptide substrates and inhibitors of thermolysin have much higher K<sub>M</sub> or K<sub>I</sub> values (Feder *et al.*, 1974). The strong reversible binding of a number of other peptide hydroxamic acids to thermolysin is also due to interaction of the hydroxamic acid functional group with the active site zinc atom (Nishino and Powers, 1978).

Evidence in support of the binding of 2-(N-bromoacetyl-N-hydroxyamino)-4-methylpentanonitrile and ClCH<sub>2</sub>CO-(N-OH)Leu-OCH<sub>3</sub> to the active site of thermolysin prior to inactivation was provided by the determination of the K<sub>I</sub> and k<sub>3</sub> values for both compounds. The competitive inhibitors P-Leu-Trp-OK and Z-Phe-OH protected thermolysin against inactivation by ClCH<sub>2</sub>CO-DL-(N-OH)Leu-OCH<sub>3</sub>. This is consistent with the modification of an active site residue since Z-Phe-OH and phosphoramidon, which is a relative of P-Leu-Trp-OH, have been shown to bind at the active site by X-ray crystallography (Kester and Matthews, 1977; Weaver *et al.*, 1977). The pH profile of alkylation of thermolysin by ClCH<sub>2</sub>CO-DL-(N-OH)Leu-OCH<sub>3</sub> closely resembled that for the hydrolysis of FA-Gly-L-Leu-NH<sub>2</sub> by the enzyme (Pangburn and Walsh, 1975) suggesting the involvement of the same residues in substrate hydrolysis and inhibition. Furthermore, the 1 to 1

stoichiometry of inactivation is consistent with modification at the active site of the enzyme.

Alkylation of three different residues (Glu-143, Tyr-157, His-231) in the active site of thermolysin could be imagined. Since there were no differences between the amino acid analyses of alkylated and nonalkylated thermolysin, the bond linking the enzyme and the inhibitor must be acid labile. This experiment points to formation of an ester between the carboxyl group of Glu-143 and the inhibitor (Fig. 6). Alkylation of either a histidine or tyrosine would have yielded a stable carboxymethyl derivative upon acid hydrolysis. Consistent with ester formation is the observation that treatment of thermolysin alkylated with  $^{14}\text{C}$ -labeled  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  was sensitive to base since 1 mM NaOH removed 99% of the incorporated label after an 8 hr treatment. Insignificant enzymatic activity (2%) was recovered due to thermolysin's instability at pH 11. Hydroxylamine (1M) also slowly displaced the inhibitor from the enzyme at pH 9.0. The slow rate is probably due to the inaccessibility of the occupied active site of the alkylated enzyme. The hydroxamic acid which is presumably formed in the reaction is enzymatically inactive since no activity toward  $\text{FA-Gly-L-Leu-NH}_2$  was recovered even though thermolysin itself maintained some activity under the conditions of the reaction.

To provide additional evidence that Glu-143 was the site of alkylation by  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$ , the labeled enzyme was degraded by CN-Br and the  $\text{F}_I$  fragment (residues 121-205) was shown to contain the ( $^{14}\text{C}$ ) inhibitor moiety. The active site His-231 and Asp-226 hydrogen bonded to it are part of the  $\text{F}_{II}$  fragment. Although  $\text{F}_I$  also contains

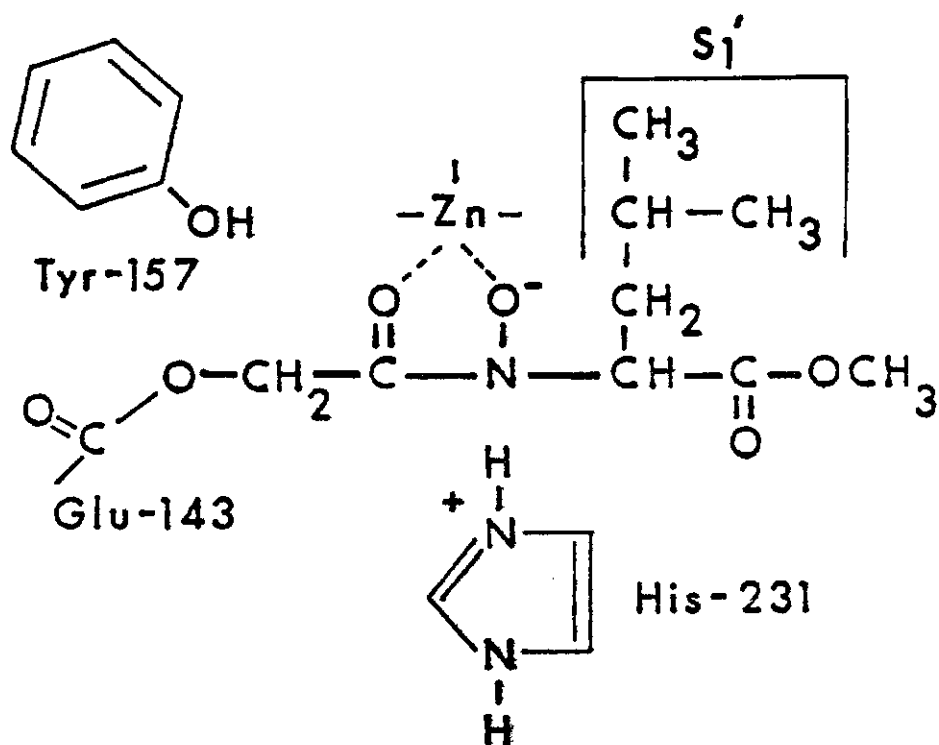


Figure 6. Schematic diagram showing the proposed interaction between the active site of thermolysin and the  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  moiety. The leucyl side chain of the inhibitor is binding to the  $S_1'$  subsite of the enzyme and the hydroxamic functional group is coordinated to the zinc atom of thermolysin either as a bidentate ligand (shown) or monodentate ligand (not shown). The active site Glu-143 has been alkylated by the inhibitor.

Tyr-157, the hydrolytic removal of the inhibitor with NaOH or 1 M  $\text{NH}_2\text{OH}$  is clearly only consistent with Glu-143 as the site of the reaction.

The specificities of 2-(N-bromoacetyl-N-hydroxyamino)-4-methylpentanonitrile and  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  were examined for a number of other proteolytic enzymes. The metalloendoproteases A and B from B. subtilis were rapidly inactivated by both compounds; whereas, the serine proteases chymotrypsin  $\text{A}_\alpha$  and subtilisin BPN' showed no loss of enzymatic activity due to the alkylating agent. The B. subtilis proteases have a substrate specificity very similar to that of thermolysin. Carboxypeptidase A was irreversibly inhibited by  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OCH}_3$  at a very slow rate ( $t_{1/2} > 3$  days), but was not inhibited by the bromoacetyl nitrile. The inhibition of carboxypeptidase may be due to slow hydrolysis of the ester in the buffer with the formation of  $\text{ClCH}_2\text{CO-DL-(N-OH)Leu-OH}$  which would probably be an excellent CPA inhibitor. At present we cannot distinguish this possibility from that involving direct inhibition of the enzyme by the ester.

The eventual extension of this new class of metalloendoprotease affinity labels to inhibit bacterial collagenase may be greatly aided by the development of the convenient and reliable pH-stat assay of clostridiopeptidase A activity using the depsipeptide Z-Gly-Pro-Leu- $\text{OCH}_2\text{CO-Pro-NH}_2$ . The assay is particularly amenable to inhibition studies on the purified collagenase. The purity of the commercial clostridiopeptidase A obtained from Sigma was not examined for the presence of isoenzymes. Lwebuga et al. (1976) have isolated four distinct collagenases from Clostridium histolyticum. All of the collagenases have comparable

catalytic activities against a series of natural and synthetic substrates and are immunologically cross-reactive. It is possible that the multiplicity of enzyme forms is, at least in part, a consequence of lysis following initial secretion from the cell.

Although thermolysin does not measurably cleave the ester bond of Z-Gly-Pro-Leu-OCH<sub>2</sub>CO-Pro-NH<sub>2</sub>, as determined with the pH-stat, it does cleave the peptide rapidly, presumably between the prolyl-leucyl amide bond, as detected by tlc methods. However, the ester bond is rapidly cleaved by porcine pancreatic elastase ( $K_m = 6.4 \pm 2.2$  mM,  $k_{cat} = 0.18 \pm 0.06$  sec<sup>-1</sup>), precluding the assay of clostridiopeptidase A activity in crude extracts. The assay gives easily reproducible results down to 10 µg/ml (~0.5 µM). To avoid excessive background hydrolysis, the assay should be performed below pH 8.5.

The inhibition studies with 1,10-phenanthroline and α-toluene-sulfonyl fluoride are consistent with clostridiopeptidase A being a metalloprotease, typically insensitive to a potent active site directed serine protease inhibitor. The chelating tripeptide Z-Gly-Pro-Leu-NHOH, based on the potent hydroxamic acid inhibitors of thermolysin, is in fact, a better inhibitor of clostridiopeptidase A than Z-Gly-Pro-Leu-OH, indicating the importance of a coordinating ligand to inhibition. The fact that the N-substituted hydroxamic acid peptides were, on the whole, very poor inhibitors of clostridiopeptidase may be an indication of the mode of inhibition by hydroxamic acid peptides in general. The substituted hydroxamic acid peptides are not able to tautomerize to the ene-diol, which presumably can form from the non substituted hydroxamic acids. The ene-diol may be the actual bidentate coordinating species to the active

site zinc for both thermolysin and clostridiopeptidase A. If this were the case, obviously a bidentate coordinating extended peptide, not requiring tautomerization, should be a very good alternative to the N-substituted hydroxamic acid peptides. A thiol containing peptide of the appropriate sequence would be a likely candidate, since, for example, 3-mercapto-2-benzylpropionyl-Ala-Gly-NH<sub>2</sub>, which is far from an optimum sequence for a substrate of clostridiopeptidase A, nevertheless was a reasonably good inhibitor of the enzyme.

## CHAPTER V

## CONCLUSIONS

In conclusion we have reported the first specific irreversible inhibitors of metalloendoproteases. The evidence is consistent with binding of the inhibitor to the active site of thermolysin with interaction of the leucyl side chain with the  $S_1'$  subsite and the hydroxamic acid functional group (as either a monodentate or bidentate ligand) with the active zinc atom. Subsequent alkylation of Glu-143 irreversibly inactivates the enzyme (Fig. 6). Since a number of non-alkylating peptide hydroxamic acids have recently been shown to reversibly bind to several metalloproteases (Nishino and Powers, 1978 and unpublished observations), haloacetyl-N-hydroxy peptides derived from the appropriate amino acids or peptides would be expected to be inhibitors of other metalloproteases.

The rapid extension of the inhibitors developed for thermolysin to the production of potent clostridiopeptidase A inhibitors should be greatly assisted by the convenient and reliable pH-stat assay of the enzyme's activity we have described. Clostridiopeptidase A, in addition to being an endopeptidase, is also an esterase, with kinetic constants for ester hydrolysis comparable with those for the analogous peptides.

## CHAPTER VI

## RECOMMENDATIONS

Solov'eva et al. (1970) reported that hydroxyproline or proline replacing leucine in several hexapeptides resulted in an increase in the rate of hydrolysis by clostridiopeptidase A. It may be possible to increase the sensitivity of the ester hydrolysis by a similar replacement. Since glycolylprolinamide is a substrate of horse liver alcohol dehydrogenase, it may be possible to couple ester hydrolysis to oxidation of the alcohol-peptide produced and follow the increase in absorption due to the production of NADH. This approach was not pursued for lack of time required to work out the problems inherent in this method.

The attachment of a fluorescent group to one end of a penta-peptide, for example, and a quenching group at the other end, might be useful in the spectrofluorimetric assay of clostridiopeptidase A activity.

Hydrolysis of the peptide would eliminate the intramolecular quenching producing a fluorescent fragment. This method, of course, has the potential of much greater sensitivity than the pH-stat method.

A potential clostridiopeptidase A affinity label would be  $\text{ClCH}_2\text{CO}-(\text{N-OH})-\text{Gly-Pro-NH}_2$ . Other potentially potent reversible inhibitors of this enzyme would be structures like:  $\text{HS-CH}_2\text{CO-Pro-NH}_2$ ,  $\text{Z-Gly-Pro-NHCH}(\underline{\text{i-Bu}})\text{CS}-(\text{N-OH})$ , and  $\text{Z-Gly-Pro-NHCH}(\underline{\text{i-Bu}})\text{CS}-(\text{N-OH})\text{Gly-Pro-NH}_2$ .

## APPENDIX

## The Amino Acid Composition of Thermolysin

Amino Acid	<u>Present Analysis</u> <sup>a</sup>		From sequence Titani <u>et al.</u> (1972b)	F <sub>I</sub> observed <sup>d</sup>	F <sub>I</sub> Titani <u>et al.</u> (1972a)
	Control	Alkylated Enzyme			
Lys	11	11	11	1.5	1
His	8	8	8	2.2	2
Arg	10	10	10	1.4	1
Asx	44	44	44	12.7	12
Thr	23	23 <sup>b</sup>	25	6.6 <sup>b</sup>	6
Ser	22	22 <sup>b</sup>	26	5.5 <sup>b</sup>	6
Glx	22	22	21	7.6	8
Pro	8	8	8	2.6	3
Gly	36	36	36	11.1	11
Ala	28	28	28	7.2	6
Cys/3	0	0	0	--	--
Val	22	22 <sup>c</sup>	22	5.4 <sup>c</sup>	6
Met	2	2	2	--	--
Ile	15	15 <sup>c</sup>	18	6.9 <sup>c</sup>	8
Leu	16	16	16	5.0 <sup>b</sup>	5
Tyr	26	26 <sup>b</sup>	28	5.7 <sup>b</sup>	5
Phe	8	8 <sup>b</sup>	10	3.1	3
Trp	--	--	3	--	--

<sup>a</sup>The values were for 24 hr hydrolysates. Calculations were based on 28 residues of Ala per molecule.

<sup>b</sup>No correction was made for decomposition of the amino acid.

<sup>c</sup>No correction was made for the incomplete hydrolysis of the amino acid.

<sup>d</sup>Calculations were based on 5 residues of Leu per molecule.

## BIBLIOGRAPHY

- Breslow, R., and Wernick, D. (1976), J. Amer. Chem. Soc. 98, 259.
- Breslow, R., and Wernick, D. (1977), Proc. Natl. Acad. Sci. U.S.A. 74, 1303
- Colman, P. M., Jansonius, J. N., and Matthews, B. W. (1972) J. Mol. Biol. 70, 701.
- Cook, A. H., and Slater, C. A. (1956), J. Chem. Soc., 4130.
- Feder, J., Brougham, L. R., and Wildi, B. S. (1974), Biochemistry 13, 1186.
- Goodman, M., and Stueben, K. C. (1962), J. Amer. Chem. Soc. 84, 1279.
- Harris, E. D., and Krane, S. M. (1974), N. Engl. J. Med. 291, 557, 605, 652.
- Hartsuch, J. A., and Lipscomb, W. N. (1971), in The Enzymes 3rd. Ed. (Boyer, P. D., ed.) Vol. 3, 1-56, Academic Press, New York.
- Hass, G. M., Govier, M. A., Grahn, D. T., and Neurath, H. (1972), Biochemistry 11, 3787.
- Hass, G. M., and Neurath, H. (1971a), Biochemistry 10, 3535.
- Hass, G. M., and Neurath, H. (1971b), Biochemistry 10, 3541.
- Kester, W. R. and Matthews, B. W. (1977a), Biochemistry 16, 2506.
- Kester, W. R. and Matthews, B. W. (1977b), J. Biol. Chem. 252, 7704.
- Kimmel, M. T., and Plummer, Jr., T. H. (1972), J. Biol. Chem., 247, 7864.
- Kitz, R., and Wilson, I. (1962), J. Biol. Chem. 237, 3245.
- Komiyama, T., Suda, H., Aoyagi, T., Takeuchi, T., and Umezawa, H. (1975), Arch. Biochem. Biophys. 171, 727.
- Lieberman, R., and Moghissi, A. A. (1970), Journal of Applied Radiation and Isotopes 21, 319.
- Lwebuga, J. S., Harper, E., and Taylor, P. (1976), Biochemistry 15, 4736.

## BIBLIOGRAPHY

(Continued)

- Matthews, B. W., Colman, P. M., Jansonius, J. N., Titani, K., Walsh, K. A., Neurath, H. (1972a), Nature (London), New Biol. 238, 41.
- Matthews, B. W., Jansonius, J. N., Colman, P. M., Schoenborn, B. P., and Dupourque, D. (1972b), Nature (London), New Biol. 238, 37.
- Neelakantan, L., and Hartung, W. H. (1958), J. Org. Chem. 23, 964.
- Nishino, N., and Powers, J. C. (1978), Biochemistry 17, 2846.
- Ohta, Y., Ogura, Y., and Wada, A. (1966), J. Biol. Chem. 241, 5919.
- Okamoto, K., Abe, H., Kuromizu, K., and Izumiya, N. (1974), Memoirs of the Faculty of Science, Kyushu University, Series C, Chemistry 9, 131.
- Pangburn, M. K., and Walsh, K. A. (1975), Biochemistry 14, 4050.
- Polonski, T., Chimiak, H. (1976), J. Org. Chem. 41, 2092
- Plummer, Jr., T. H. (1971), J. Biol. Chem. 246, 2930.
- Plummer, Jr., T. H., and Kimmel, M. T. (1969), J. Biol. Chem. 245, 5246.
- Powers, J. C. (1977), in Chemistry and Biochemistry of Amino Acids, Peptides and Proteins (Weinstein, R., ed.) Vol. 4, 65-178, Dekker, New York.
- Rasnick, D. W. (1978), Fed. Proc. 37, 99.
- Rasnick, D. W., and Powers, J. C. (1978), Biochemistry 17, 0000
- Rosen, H. (1957), Aech. Biochem. Biophys. 67, 10.
- Schechter, I., and Berger, A. (1967), Biochem. Biophys. Res. Commun. 27, 157.
- Schmid, M. F., and Herriott, J. R. (1976), J. Mol. Biol. 103, 175.
- Shimohigashi, Y., Lee, S., Aoyagi, H., Kato, T., and Izumiya, N. (1977), Int. J. Peptide Protein Res. 10, 197.
- Sokolovsky, M., and Zisapel, N. (1974), Israel J. Chem. 12, 631.
- Solov'eva, N. I., Orekhovich, V. N., Shibnev, V. A., and Lazareva, A. V. (1970), Biochemia 35, 579.

## BIBLIOGRAPHY

(Continued)

- Suda, H., Aoyagi, T., Takenchi, T., and Umezawa, H. (1973), J. Antibiot. (Tokyo) 26, 621.
- Titani, K., Hermodson, M. A., Ericsson, H., Walsh, K. A., and Neurath, H. (1972a), Biochemistry 11, 2427.
- Titani, K., Hermodson, M. A., Ericsson, H., Walsh, K. A., and Neurath, H. (1972b), Nature (London), New Biol. 238, 35.
- Walsh, K. A., Burstein, Y., and Pangburn, M. K. (1975), Methods Enzymol. 34, 435.
- Weaver, L. H., Kester, W. R., and Matthews, B. W. (1977), J. Mol. Biol. 114, 119.
- Wilcox, P. E. (1970), Methods Enzymol. 19, 64.
- Williams, S. F. (1952), J. Amer. Chem. Soc. 74, 4708.
- Yagisawa, S., Morita, F., Nagai, Y., Noda, H., and Ogura, Y. (1965), J. Biochem. 58, 407.
- Zisapel, N., and Sokolovsky, M. (1974), Biochem. Biophys. Res. Comm. 58, 951