in solution is characterized by a dynamic equilibrium of multiple conformational states. Detailed examination of the kinetic and equilibrium properties of these states has and will continue to provide new insights into the mechanism of action.

References

Alter, G. M., and Vallee, B. L. (1978), *Biochemistry 17,* 2212.

French, T. C., Yu, N.-T., and Auld, D. S. (1974), *Biochemistry 13,* 2877.

Hammes, G. G. (1974), in *Investigation of Rates and Mechanisms of Reactions* (Hammes, G. G., Ed.) Part 2, 3rd ed, p 147, Wiley, New York, N.Y.

Hammes, G. G., and Schimmel, P. R. (1970), *Enzymes, 3rd Ed., 2,* 67.

Harrison, L. W., Auld, D. S., and Vallee, B. L. (1975a), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3930.

Harrison, L. W., Auld, D. S., and Vallee, B. L. (1975b), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4356.

Johansen, J. T., and Vallee, B. L. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2532.

Johansen, J. T., and Vallee, B. L. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2006.

Johansen, J. T., and Vallee, B. L. (1975), *Biochemistry 14,* 649.

Johansen, J. T., Klyosov, A. A., and Vallee, B. L. (1976), *Biochemistry 15,* 296.

Thiers, R. E. (1957), *Methods Biochem. Anal. 5,* 273.

Vallee, B. L., Riordan, J. F., Johansen, J. T., and Livingston, D. M. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 517.

Active Site Directed Irreversible Inhibition of Thermolysin†

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ABSTRACT: Thermolysin is irreversibly inhibited at pH 7.2 by $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 and 2-(*N*-bromoacetyl-*N*-hydroxyamino)-4-methylpentanonitrile. 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}C-labeled $ClCH_2CO$-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 NH_2OH, observations which support the existence of an ester linkage between the enzyme and inhibitor. Degradation of thermolysin alkylated with ^{14}C-labeled $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 with CNBr demonstrated that the F_I 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 $BrCH_2CO$-Phe-OCH_3, $BrCH_2CO$-L-MeLeu-OCH_3, and $BrCH_2CO$-L-MeLeu-L-Ala-OCH_3 did not react with enzyme. The inhibitor $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 exhibited considerable specificity and would not inhibit the serine proteases chymotrypsin A_α 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*-hydroxypeptides with the appropriate amino acid or peptide structures should be useful for the inhibition of other metalloproteases.

Thermolysin (EC 3.4.24.4) is a neutral metalloendoprotease which requires a zinc atom at the active site for enzymatic activity. The amino acid sequence (Titani et al., 1972b) 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 & Powers, 1978) and phosphoramidate (Kam & Powers, unpublished results) functional groups have recently been synthesized. The development of these compounds is part of a continuing effort to produce inhibitors for the biologically important metalloendoproteases. A particular example is the enzyme collagenase which has been implicated in the debilitating disease arthritis (Harris & Krane, 1974). Specific irreversible inhibitors of metalloendoproteases could be of potential therapeutic value as well as aiding in the study of the active sites of these enzymes.

Only a limited number of specific active site directed irreversible inhibitors of metalloproteases have been reported. The available inhibitors are directed against either carboxypeptidase A or B. These enzymes are similar to thermolysin both in size and in the catalytic mechanism which requires an essential zinc for enzymatic activity, but differ in cleaving the amide bonds at the carboxy terminal end of peptides. *N*-Bromoacetyl-L-*N*-methylphenylalanine has been shown to modify the essential glutamic acid-270 of bovine carboxypeptidase A_γ^{Leu} (Hass & Neurath, 1971a,b) and bovine carboxypeptidase B (Hass et al., 1972). Carboxypeptidase B is also modified by *N*-bromoacetyl-D-arginine, a reagent that stoichiometrically reacts with the active site glutamic acid residue (Plummer, 1971; Kimmel & Plummer, 1972; Sokolovsky & Zisapel, 1974). The side chains of Tyr-248 and a methionine residue of carboxypeptidase B are modified respectively by bromoacetamidobutylguanidine (Plummer and Kimmel, 1969) and *N*-bromoacetyl-*p*-aminobenzyl-L-succinic acid (Zisapel & Sokolovsky, 1974). To our knowledge no such active site di-

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rected inhibitors are available for any other metalloproteases.

In our initial search for inhibitors we have utilized thermolysin as a model metalloendoprotease since detailed structural information is available for this enzyme. In this paper we report an unsuccessful attempt to extend the class of compounds developed by Hass & Neurath to 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. This class of inhibitor should be useful for the inhibition of other members of the metalloprotease family.

Materials and Methods

The following enzymes were obtained from the Sigma Chemical Co.: 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. Crystalline chymotrypsin A_α (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 ($HONHCOCH(CH_2C_6H_5)CO$-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 and bromoacetyl bromide (Aldrich Chemical Co.); furylacryloylglycyl-L-leucinamide (FA-Gly-Leu-NH_2)[1] (Vega-Fox Biochemicals); *N-tert*-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-^{14}C]chloroacetic acid (New England Nuclear); cyanogen bromide (Eastman Organic Chemicals) was stored in a sealed desiccator at 4 °C 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 Co. 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) was synthesized by Dr. Chih-Min Kam of our group.

Analytical Procedures. Peptidase activities 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_2 (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^{-1} cm^{-1} was used for thermolysin (Ohta et al., 1966; Titani et al., 1972b). The concentrations of neutral proteases A and B and chymotrypsin A_α were similarly determined, $\epsilon_{280}^{1\%} = 14.8$ and 14.7 cm^{-1} (Walsh et al., 1975) and $\epsilon_{282}^{M} = 50\,000$ M^{-1} cm^{-1} (Wilcox, 1970) respectively. 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 a toluene-Triton X-100 solution (Lieberman & Moghissi, 1970). Elemental analysis was performed by Atlantic Microlab, Atlanta, Ga. Infrared spectra were performed on a Perkin-Elmer 457 instrument. Proton magnetic resonance (^{1}H NMR) 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-h hydrolysates (6 N 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 (1:2, 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 °C with stirring 30 min at −5 °C. 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 $NaHCO_3$, 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 °C, R_f^1 0.92, R_f^2 0.77. This compound had been prepared by a different method, mp 80–82 °C (Williams, 1952).

The ^{1}H NMR ($CDCl_3$) spectrum showed peaks at δ 7.2 (6 H, s, Ph + N*H*), 4.8 (1 H, m, —(N)C*H*–), 3.8 (2 H, s, $BrCH_2$–), 3.7 (3 H, s, CH_3–O), 3.2 (2 H, d, Ph–CH_2–).

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 h. 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 $NaHCO_3$, 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^{-1} (amide CO).

The ^{1}H NMR ($CDCl_3$) spectrum showed peaks at δ 5.2 (1 H, t, N–C*H*–), 4.0 (2 *H*, s, $BrCH_2$), 3.7 (3 H, s, CH_3–O), 3.0 (3 H, s, CH_3N), 2.0–1.2 (3 H, m, CH_2C*H*), 0.9 (6 H, d, CH(CH_3)$_2$).

Anal. Calcd for $C_{10}H_{18}BrNO_3$: 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 ^{1}H NMR ($CDCl_3$) spectrum showed peaks at δ 6.9 (1 H, d, NH), 5.2 (1 H, t, NC*H*CH_2), 4.4 (1 H, m, –(NH)-C*H*CH_3), 4.0 (2 H, s, $BrCH_2$), 3.8 (3H, s, CH_3–O), 3.0 (3 H, s, CH_3–N), 1.7 (3 H, m, CH_2C*H*(CH_3)$_2$), 1.2 (3 H, d, –(NH)CHCH_3), 0.9 (6 H, d, (CH_3)$_2$).

2-(N-Bromoacetyl-DL-N-hydroxyamino)-4-methylpentanonitrile. To DL-2-hydroxyamino-4-methylpentanonitrile (1.73 g, 13.5 mmol) (Neelakantan & Hartung, 1958) in 20 mL of acetonitrile was added bromoacetyl bromide (1.36 g, 6.75

[1] Abbreviations used: Z, benzyloxycarbonyl; $BrCH_2CO$-L-Phe-OCH_3, *N*-bromoacetyl-L-phenylalanine methyl ester; $BrCH_2CO$-L-MeLeu-OCH_3, *N*-bromoacetyl-L-*N*-methylleucine methyl ester; $BrCH_2CO$-L-MeLeu-L-Ala-OCH_3, *N*-bromoacetyl-L-*N*-methylleucyl-L-alanine methyl ester; $ClCH_2CO$-DL-(N-OH)Leu-OCH_3, *N*-chloroacetyl-DL-*N*-hydroxyleucine methyl ester; DMF, dimethylformamide; FA-Gly-L-Leu-NH_2, 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(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane.

mmol) at room temperature. The reaction was stopped after 2 h. The acetonitrile was removed by evaporation and the residue was taken up in 100 mL of chloroform, washed with 50 mL of 0.1 M $NaHCO_3$, 50 mL of 1.0 M HCl, and 50 mL of water, and then dried over anhydrous magnesium sulfate. The $CHCl_3$ 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 °C and 0.05 mm pressure. The IR (neat) had absorption bands at 3220 (OH), 2250 (CN), and 1655 cm^{-1} (amide CO); R_f^1 0.80, R_f^2 0.55.

The ^1H NMR ($CDCl_3$) spectrum showed peaks at δ 5.6 (1 H, t, -CH-CN), 4.2 (2 H, s, $BrCH_2$-), 2.2–1.5 (3 H, m, CH_2CH), 0.9 (6 H, d, $CH(CH_3)_2$).

N-Chloroacetyl-DL-N-hydroxyleucine methyl ester was prepared by the procedure of Cook & Slater (1956): mp 80–80.5 °C (lit. mp 81 °C).

The ^1H NMR ($CDCl_3$) spectrum showed peaks at δ 5.3 (1 H, t, N-CH), 4.4 (2 H, s, $ClCH_2$-), 3.8 (3 H, s, CH_3-O), 2.2–1.4 (3 H, m, CH_2CH), 0.9 (6 H, d, $CH(CH_3)_2$).

Anal. Calcd for $C_9H_{16}ClNO_4$: C, 45.48; H, 6.78; N, 5.89. Found: C, 45.50; H, 6.82; N, 5.88.

N-[1-^{14}C]Chloroacetyl-DL-N-hydroxyleucine Methyl Ester. [1-^{14}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 °C were added pyridine (150 μL) and thionyl chloride (150 μL). The reaction was protected with a calcium chloride drying tube and allowed to proceed 1 h at room temperature. The solution was filtered and to the filtrate at 0 °C were added 200 mg of solid $NaHCO_3$ and DL-*N*-hydroxyleucine methyl ester (410 mg, 2.55 mmol) (Cook & 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 $NaHCO_3$ solution, 15 mL of 1.0 M HCl, and 15 mL of water and 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 of ether. Crystallization was initiated by the addition of petroleum ether. The overall yield was 196 mg (49%), mp 80–80.5 °C. 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 $BrCH_2CO$-L-Phe-OCH_3, $BrCH_2CO$-L-MeLeu-OCH_3 and $BrCH_2CO$-L-Me-Leu-L-Ala-OCH_3. 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 $CaCl_2$, pH 7.2) at 25 °C was added 50 μL of a dimethylformamide (DMF) solution of the alkylating agent. The final concentrations were 0.19 μM and 0.10 mM for the enzyme and alkylating agent, respectively. Incubation was continued at least 48 h. Aliquots (50 μL) 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-hydroxyamino)-4-methylpentanonitrile. To 1-mL solutions of thermolysin in the above Tris buffer at 25 °C were added 50 μL 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 μM, and the final DMF concentration was 5%. Aliquots (50 μL) 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 $ClCH_2CO$-DL-(N-OH)Leu-OCH_3. To 1-mL solutions of the appropriate enzyme at 25 °C were added 50 μL 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 μM; 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 Carboxypeptidase A with $ClCH_2CO$-DL-(N-OH)Leu-OCH_3. To a 1-mL solution of carboxypeptidase A (0.46 mg/mL, 1.0 M NaCl, 5 mM K_2HPO_4, pH 7.5) was added 50 μL of a methanolic solution of the alkylating agent to give a final inhibitor concentration of 1.0 mM. This solution was incubated at 25 °C for 3 days along wth a control solution containing the enzyme and 50 μL of methanol. Periodically 50-μL 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_α and Subtilisin BPN' with $ClCH_2CO$-DL-(N-OH)Leu-OCH_3. A 1-mL solution of chymotrypsin A_α (0.10 M Hepes, 1.0 M NaCl, pH 7.5) and 50 μL of a methanolic solution of the alkylating agent were mixed together to give final enzyme and alkylating agent concentrations of 0.93 μM and 1.0 mM, respectively. This solution was incubated at 25 °C for 3 days along with a control lacking the alkylating agent. Periodically 50-μL aliquots of these solutions were assayed for residual enzyme activity by their addition to 2-mL solutions containing 50 μM 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_α to give a final enzyme concentration of 95 μg/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 6.50–7.70), and Tris (pH 8.00–8.95); all were 0.10 M and contained 0.01 M $CaCl_2$.

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

Active Site Protection of Thermolysin. Thermolysin (4.4 μM, Tris buffer, pH 7.2, 5% DMF) was incubated at 25 °C 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 & Matthews, 1977). Periodically aliquots were assayed for residual enzyme activity.

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

Incorporation of N-[1-^{14}C]Chloroacetyl-DL-N-hydrox-

yleucine Methyl Ester into Thermolysin. A 15-mL solution of purified thermolysin (20 mg) was treated with a 1-mL solution of [^{14}C]$ClCH_2CO$-(N-OH)Leu-OCH_3 (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 h at room temperature; however, complete loss of enzymatic activity toward FA-Gly-L-Leu-NH_2 was achieved after only 10 min.

Inactivated thermolysin was separated from the excess alkylating agent by passage through a 1 × 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 four peptide containing fractions was measured.

A 2-mL sample of a 7.72 μM solution of [^{14}C]thermolysin was dialyzed against 1.0 mM NaOH for 10 h at 25 °C. Periodically the radioactivity in the dialysis tubing was measured on 100-μL aliquots. A 2.5-mL sample from the same stock [^{14}C]thermolysin solution was dialyzed against a 1.0 M hydroxylamine solution (0.1 M Tris, 1.0 M NaCl, 0.01 M $CaCl_2$, 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.

Cyanogen bromide cleavage of [^{14}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 × 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 h. An aliquot (200 μL) from each fraction was added to 800 μL of water and the radioactivity present measured. The individual fragment peptides were purified by the method of Titani et al. (1972a).

Results

Haloacetyl Amino Acid Esters. Earlier studies with carboxypeptidase A (Hass & 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.[2] Thermolysin, however, does not like a terminal carboxy group. Therefore the compounds $BrCH_2CO$-L-Phe-OCH_3 and $BrCH_2CO$-L-MeLeu-OCH_3, containing a favorable P_1' residue and a blocked carboxy group, were synthesized as potential thermolysin inhibitors. However, neither of these compounds inhibited thermolysin after 48-h 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 FA-Gly-L-Leu-NH_2. We then decided to extend the peptide chain of the inhibitor and synthesized $BrCH_2CO$-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 reversible inhibitors of thermolysin with K_I values in the μM range (Nishino & 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 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 $CaCl_2$) containing 5% DMF at 25 °C.

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 ($ClCH_2CO$-DL-(N-OH)Leu-OCH_3) had been synthesized by Cook & 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 & Wilson, 1970). The observed rate constant

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_3} E\text{-}I \qquad (1)$$

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

$$\frac{1}{k_{obsd}} = \frac{K_I}{k_3[I]} + \frac{1}{k_3} \qquad (2)$$

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 $1/k_{obsd}$ vs. $1/[I]$. A plot of $1/k_{obsd}$ vs. $1/[I]$ for the irreversible inactivation of thermolysin at pH 7.2 by $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 using the least-squares method gave K_I = 7.5 ± 2.2 mM and $k_3 = 7.5 \pm 2.0 \times 10^{-3}$ s^{-1} ([I] = 10.5–2.1 mM). A similar plot for thermolysin and 2-(*N*-bromoacetyl-*N*-hydroxyamino)-4-methylpentanonitrile gave K_I = 0.80 ± 0.36 mM and $k_3 = 7.4 \pm 3.3 \times 10^{-3}$ s^{-1} ([I] = 0.87–0.12 mM). The limiting rates of inactivation (k_3) were essentially identical for the two compounds, but the bromoacetylnitrile was bound tenfold more tightly by the enzyme.

pH Dependence of Inactivation. The pH dependence of the irreversible inactivation of thermolysin by $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 was bell shaped (Figure 1) and similar to the pH profile of catalysis reported by Pangburn & Walsh (1975). The pH optimum for alkylation was around 7.5.

Active Site Protection of Thermolysin. The inactivation of thermolysin by $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 in the presence and absence of the competitive inhibitor Z-Phe-OH (K_I = 0.51 mM) is shown in Figure 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 &

[2] The nomenclature used for the individual amino acid residues (P_1, P_1', P_2', etc.) of an inhibitor and for the subsites (S_1, S_1', S_2', etc.) of the enzyme is that of Schechter & Berger (1967).

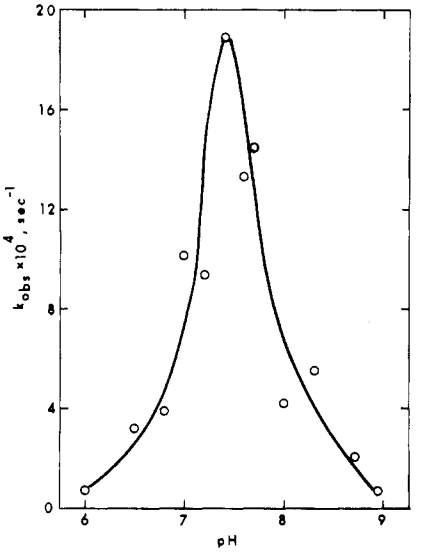

FIGURE 1: pH dependence of the inactivation of thermolysin (4.2 μM) by $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 (2.3 mM). The buffers used are described in the text.

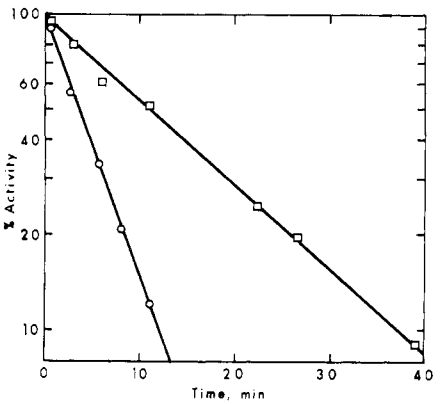

FIGURE 2: Active site protection against inactivation. Thermolysin (4.4 μM) in 0.10 M Tris, 0.01 M $CaCl_2$, pH 7.2, containing 5% DMF was incubated with $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 (4.5 mM) in the presence (□) and absence (○) of the competitive inhibitor Z-Phe-OH (1.7 mM). The enzyme was assayed for residual activity as described in the text.

Matthews, 1977). The k_{obsd} in the presence of inhibitor was 1.0×10^{-3} s^{-1}. The value calculated from the equation $k_{obsd} = k_3[I]/(K_I(1 + [I']/K_I') + [I])$, where I is the irreversible inhibitor and I' is Z-Phe-OH, was 0.90×10^{-3} s^{-1}.

When thermolysin was treated with as little as a 12-fold molar excess of the competitive inhibitor P-Leu-Trp-OK ($K_I \sim 0.1$ μM) (Komiyama et al., 1975; Kam & Powers, unpublished results) immediately prior to incubating 1.5 h in the presence of $ClCH_2CO$-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 three metalloendoproteases by $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 (8.4 mM) was made in 0.10 M Tris, 0.01 M $CaCl_2$, pH 7.2 buffer containing 5% DMF at 25 °C. The $k_{obsd}/[I]$ values of 400, 5.4, and 6.4 M^{-1} s^{-1} were observed respectively for thermolysin, neutral protease A, and neutral protease B. Neutral proteases A and B from *B. subtilis* were irreversibly inhibited nearly two orders of magnitude slower than thermolysin. Neutral protease B hydrolyzes FA-Gly-L-Leu-NH_2 three times faster than neutral protease A,

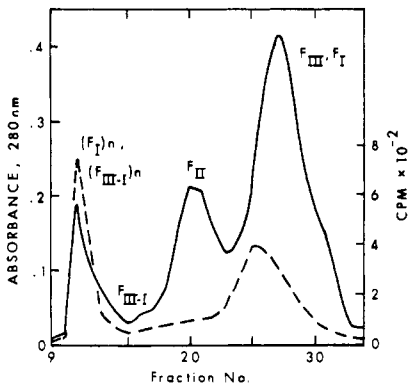

FIGURE 3: Separation of the cyanogen bromide fragments of thermolysin on a 1 × 50 cm column of Bio-Gel P-100 equilibrated and eluted with 1.0 M formic acid. Fractions (1.8 mL) were monitored both at 280 nm (solid line) and for radioactivity (broken line). Peaks were labeled as described by Titani et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

but both these enzymes were alkylated at about the same rate.

Carboxypeptidase A was irreversibly inhibited by $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 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_α and subtilisin BPN' showed no measurable loss of their enzymatic activities after 3 days of incubation in the presence of $ClCH_2CO$-(N-OH)Leu-OCH_3 when compared with their controls.

Incorporation of N-[1-^{14}C]Chloroacetyl-DL-N-hydroxyleucine Methyl Ester into Thermolysin. The incorporation of carbon-14 labeled $ClCH_2CO$-DL-(N-OH)-Leu-OCH_3 into thermolysin resulted in 0.97 ± 0.03 inhibitor molecule 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 h. 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 $CaCl_2$ the release of radioactivity was quite slow ($t_{1/2}$ = 175 h). There was no recovery of thermolysin's ability to hydrolyze FA-Gly-L-Leu-NH_2; whereas a control with nonalkylated thermolysin retained 18% enzymatic activity after 216 h in the presence of 1.0 M hydroxylamine and pH 9.0.

Amino Acid Analysis of Alkylated Thermolysin. Thermolysin was alkylated with $ClCH_2CO$-(N-OH)Leu-OCH_3 and an amino acid analysis was performed and compared with that for unlabeled enzyme (see paragraph concerning supplementary material at the end of this paper). 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. Carboxymethylhistidine and carboxymethyltyrosine did not appear in the amino acid analysis of alkylated thermolysin.

Cyanogen Bromide Cleavage of Thermolysin Alkylated with N-[1-^{14}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 Figure 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 inhibitor molecule attached.

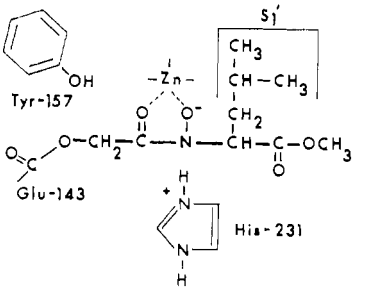

FIGURE 4: Schematic diagram showing the proposed interaction between the active site of thermolysin and the $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 moiety. The leucyl side chain of the inhibitor is binding to the S_1' subsite of the enzyme and the hydroxamic acid functional group is coordinated to the zinc atom of thermolysin either as a bidentate ligand (shown) or monodentate ligand (not shown). The active site Glu-143 has been alkylated by the inhibitor.

Discussion

The molecular structures of carboxypeptidase A (Hartsuck & Lipscomb, 1971), carboxypeptidase B (Schmid & 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 or 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 & Neurath, 1971a,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 ($BrCH_2CO$-L-Phe-OCH_3, $BrCH_2CO$-L-MeLeu-OCH_3, and $BrCH_2CO$-L-MeLeu-L-Ala-OCH_3) showed no indication of being thermolysin inhibitors. It is difficult to explain why the first compound ($BrCH_2CO$-L-Phe-OCH_3) was not an inhibitor since Gly-Phe-NH_2 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 $BrCH_2CO$-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_1' 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 $BrCH_2CO$-L-MeLeu-L-Ala-OCH_3 had no effect on the inhibition.

Replacement of the P_1' 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 $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 bound to and rapidly irreversibly inactivated thermolysin. The increased binding of the *N*-hydroxy peptides to thermolysin is probably due to coordinating of the hydroxamic acid functional group to the active site zinc atom of thermolysin. The K_I of the bromoacetylnitrile is 0.80 mM and that of $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 is 7.5 mM, while most dipeptide substrates and inhibitors of thermolysin have much higher K_M or K_I 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 & Powers, 1978).

Evidence in support of the binding of 2-(*N*-bromoacetyl-*N*-hydroxyamino)-4-methylpentanonitrile and $ClCH_2CO$-(N-OH)Leu-OCH_3 to the active site of thermolysin prior to inactivation was provided by the determination of the K_I and k_3 values for both compounds. The competitive inhibitors P-Leu-Trp-OK and Z-Phe-OH protected thermolysin against inactivation by $ClCH_2CO$-DL-(N-OH)Leu-OCH_3. 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-OK, have been shown to bind at the active site by X-ray crystallography (Kester & Matthews, 1977; Weaver et al., 1977). The pH profile of alkylation of thermolysin by $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 closely resembled that for the hydrolysis of FA-Gly-L-Leu-NH_2 by the enzyme (Pangburn & 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 (Figure 4). 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}C-labeled $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 was sensitive to base since 1 mM NaOH removed 99% of the incorporated label after an 8 h treatment. Insignificant enzymatic activity (2%) was recovered due to thermolysin's instability at pH 11. Hydroxylamine (1 M) 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 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 $ClCH_2CO$-DL-(N-OH)Leu-OCH_3, the labeled enzyme was degraded by CNBr and the F_I fragment (residues 121–205) was shown to contain the ^{14}C-labeled inhibitor moiety. The active site His-231 and Asp-226 hydrogen bonded to it are part of the F_{II} fragment. Although F_I also contains Tyr-157, the hydrolytic removal of the inhibitor with NaOH or 1 M NH_2OH is clearly only consistent with Glu-143 as the site of the reaction.

The specificities of 2-(*N*-bromoacetyl-*N*-hydroxyamino)-4-methylpentanonitrile and $ClCH_2CO$-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 A_α 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 $ClCH_2CO$-DL-(N-OH)Leu-OCH_3 at a very slow rate ($t_{1/2}$ > 3 days), but was not touched by the bromoacetylnitrile. The inhibition of carboxypeptidase may be due to slow hydrolysis of the ester in the buffer with the formation of $ClCH_2CO$-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.

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 (Figure 4). Since a number of nonalkylating peptide hydroxamic acids have recently been shown to reversibly bind to several metalloproteases (Nishino & 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.

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Supplementary Material Available

The amino acid analysis of alkylated thermolysin and the alkylated F_I fragment (1 page). Ordering information is given on any current masthead page.

References

Colman, P. M., Jansonius, J. N. & Matthews, B. W. (1972) *J. Mol. Biol. 70,* 701.
Cook, A. H., & Slater, C. A. (1956) *J. Chem. Soc.,* 4130.
Feder, J., Brougham, L. R., & Wildi, B. S. (1974) *Biochemistry 13,* 1186.
Harris, E. D., & Krane, S. M. (1974) *N. Engl. J. Med. 291,* 557, 605, 652.
Hartsuch, J. A., & Lipscomb, W. N. (1971) *Enzymes, 3rd Ed. 3,* 1–56.
Hass, G. M., & Neurath, H. (1971a) *Biochemistry 10,* 3535.
Hass, G. M., & Neurath, H. (1971b) *Biochemistry 10,* 3541.
Hass, G. M., Govier, M. A., Grahn, D. T., & Neurath, H. (1972) *Biochemistry 11,* 3787.
ester, W. R., & Matthews, B. W. (1977) *Biochemistry 16,* 2506.
Kimmel, M. T., & Plummer, T. H., Jr. (1972) *J. Biol. Chem. 247,* 7864.
Kitz, R., & Wilson, I. (1962) *J. Biol. Chem. 237,* 3245.
Komiyama, T., Suda, H., Aoyagi, T., Takeuchi, T., & Umezawa, H. (1975) *Arch. Biochem. Biophys. 171,* 727.
Lieberman, R., & Moghissi, A. A. (1970) *J. Appl. Radiat. Isotop. 21,* 319.
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., & Dupourque, D. (1972b) *Nature (London), New Biol. 238,* 37.
Neelakantan, L., & Hartung, W. H. (1958) *J. Org. Chem. 23,* 964.
Nishino, N., & Powers, J. C. (1978) *Biochemistry 17,* 2846.
Ohta, Y., Ogura, Y., & Wada, A. (1966) *J. Biol. Chem. 241,* 5919.
Okamoto, K., Abe, H., Kuromizu, K., & Izumiya, N. (1974) *Mem. Fac. Sci., Kyushu Univ., Ser. C 9,* 131.
Pangburn, M. K., & Walsh, K. A. (1975) *Biochemistry 14,* 4050.
Plummer, Jr., T. H. (1971) *J. Biol. Chem. 246,* 2930.
Plummer, Jr., T. H., & Kimmel, M. T. (1969) *J. Biol. Chem. 245,* 5246.
Powers, J. C. (1977) in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins* (Weinstein, B., Ed.) Vol. 4, pp 65–178, Marcel Dekker, New York, N.Y.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27,* 157.
Schmid, M. F., & Herriott, J. R. (1976) *J. Mol. Biol. 103,* 175.
Sokolovsky, M., & Zisapel, N. (1974) *Isr. J. Chem. 12,* 631.
Titani, K., Hermodson, M. A., Ericsson, H., Walsh, K. A., & Neurath, H. (1972a) *Biochemistry 11,* 2427.
Titani, K., Hermodson, M. A., Ericsson, H., Walsh, K. A., & Neurath, H. (1972b) *Nature (London), New Biol. 238,* 35.
Walsh, K. A., Burstein, Y., & Pangburn, M. K. (1975) *Methods Enzymol. 34,* 435.
Weaver, L. H., Kester, W. R., & Matthews, B. W. (1977) *J. Mol. Biol. 114,* 119.
Wilcox, P. E. (1970) *Methods Enzymol. 19,* 64.
Williams, S. F. (1952) *J. Am. Chem. Soc. 74,* 4708.
Zisapel, N., & Sokolovsky, M. (1974) *Biochem. Biophys. Res. Commun. 58,* 951.