Inhibition of Thermolysin and Carboxypeptidase A by Phosphoramidates[†]

Chih-Min Kam, Norikazu Nishino, and James C. Powers*

ABSTRACT: A series of N-phosphorylated dipeptides and amino acid derivatives have been synthesized. These compounds, which are analogues of phosphoramidon, are competitive inhibitors of thermolysin. The simple phosphoramidate P-Leu-NH₂ was an excellent inhibitor with a K_1 of 1.3 μ M, while H-Leu-NH₂ is ca. 10^3 times poorer. O-Methylphosphoryl-Leu-NH₂ inhibits thermolysin 100 times poorer than P-Leu-NH₂. O-Methyl-O-phenylphosphoryl-Leu-NH₂ and N-ethylphosphoramidate do not show any inhibition at 3 mM. It is suggested that the N-phosphorylleucyl moiety plays the major role in the inhibition and accounts for at least 65% of the free energy of binding of phosphoramidon. The Nphosphorylated dipeptides P-Ile-Ala-OH, P-Leu-Phe-OH, and P-Leu-Trp-OH inhibited thermolysin up to 85-fold better than P-Leu-NH₂. A hydrophobic P_1' amino acid is required since P-Ala-Ala-OH binds 240-fold less tightly than P-Ile-Ala-OH.

Metalloproteases are a family of enzymes characterized by the presence of a catalytically essential zinc atom in their active sites. Two widely studied members of this family are thermolysin and carboxypeptidase A. Thermolysin is the thermostable metalloendoprotease produced by Bacillus thermoproteolyticus and is representative of a number of zinc-requiring microbial neutral proteases. Thermolysin exhibits a specificity toward peptide bonds in which the amino group is contributed by amino acid residues having hydrophobic side chains such as isoleucine, leucine, and phenylalanine (Morihara, 1967; Morihara & Oka, 1968; Morihara et al., 1968; Feder & Schuck, 1970). Carboxypeptidase A in contrast is an exopeptidase with a preference for carboxylterminal aromatic and hydrophobic amino acid residues. The crystal structures of both enzymes have been determined, and the enzymes share many common features in their active sites (Kester & Matthews, 1977b).

Recently phosphoramidon, N-(α -L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan, has been isolated from a culture filtrate of Streptomyces tanashiensis (MOD 706-Y4) and shown to be a potent inhibitor of thermolysin (Suda et al., 1972; Umezawa et al., 1972). Phosphoramidon showed little inhibitory effect against other proteases such as trypsin, α -chymotrypsin, papain, and pepsin (Suda et al., 1972; Komiyama et al., 1975a,b). A crystallographic study of the thermolysin-phosphoramidon complex has demonstrated that the inhibitor binds at the active site of the enzyme and resembles the presumed transition state for thermolysin-catalyzed peptide bond hydrolysis (Weaver et al., 1977). No phosphoryl amino acids or peptides have been reported to be inhibitors of carboxypeptidase A, but several dicarboxylic acids with aromatic side chains have been found to be strong competitive inhibitors (Byers & Wolfenden, 1972). One inhibitor, Lbenzylsuccinic acid, has been shown to bind at the active site An aromatic residue at P_2 also contributes to the binding since the K_{I} of P-Leu-Trp-OH or P-Leu-Phe-OH is ca. 200 times lower than that of P-Ile-Ala-OH. The chelation of the active site zinc atom by the phosphoryl group and a hydrophobic interaction between the side chain of the P_1' amino acid residue and the S_1 subsite of the enzyme are the primary factors responsible for the inhibition. Additional interactions with Tyr-153 and His-231 are proposed to account for the lower K_1 values of phosphoramidates when compared with their monoalkyl esters. N-Phosphorylphenylalanine (P-Phe-OH) is a good inhibitor of carboxypeptidase A, and other Nphosphoryl derivatives, P-Leu-NH₂, P-Leu-Trp-OH, and P-NH-Et, also show weaker inhibition. Phosphoramidate derivatives of the appropriate amino acids or peptides should be useful as reversible inhibitors or affinity ligands for other metalloproteases.

of carboxypeptidase A, probably due to chelation of a carboxylate group of the inhibitor with the zinc atom of the enzyme (Byers & Wolfenden, 1973).

In this paper, we report the synthesis of several N-phosphoryl peptides and amino acid derivatives and show which elements of the phosphoramidon structure are responsible for tight binding to thermolysin. In addition, several phosphoramidates are shown to inhibit carboxypeptidase A. This indicates that this class of useful inhibitors could be extended to most other metalloproteases.

Materials and Methods

Crystalline thermolysin was obtained from Sigma and Calbiochem. Bovine carboxypeptidase A was purchased from Sigma. The substrates, FA-Gly-Leu- NH_2^{1} (Vega-Fox) and Z-Gly-Phe-OH (Pierce), were used without further purification. Samples of phosphoramidon were obtained from Drs. T. Aoyagi (Tokyo, Japan), K. Walsh (University of Washington), and W. Troll (New York University). Reagent grade solvents and chemicals were used throughout these studies. Dibenzyl phosphite and dibenzyl phosphochloridate were prepared according to previously described methods (Li & Eakin, 1955; Atherton et al., 1948).

Phosphorylleucinamide (P-Leu-NH₂). Dibenzylphosphorylleucinamide was prepared by coupling (BzlO)₂POCl with H-Leu-NH₂·HCl (Li, 1952) and had mp 116–117 °C; NMR (CDCl₃) δ 7.3 (s, 10 H, Ph), 5.0 (d, 4 H, PhCH₂OP), 3.9 (m, 1 H, NCHCO), 1.6 (b, 3 H, CH₂CH), 0.85 (d, 6 H, (CH₃)₂CH). Anal. Calcd for C₂₀H₂₇N₂O₄P: C, 61.54; H, 6.93; N, 7.15. Found: C, 61.42; H, 6.91; N, 7.12.

Dibenzylphosphorylleucinamide (0.5 g) was dissolved in 50 mL of isopropyl alcohol and hydrogenated at atmospheric pressure by using 10% Pd on charcoal as a catalyst. After 30

[†]From the School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332. *Received August 25, 1978; revised manuscript received March 8, 1979.* This research was supported by a grant from the U.S. Public Health Service (HL 18679).

¹ Abbreviations used: FA-Gly-Leu-NH₂, furylacryloylglycyl-Lleucinamide; Z, benzyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Abz, 2-aminobenzoyl; Nba, 4-nitrobenzylamide; (BzlO)₂POCl, dibenzyl phosphochloridate; P, phosphoryl group [(HO)₂PO- or salt]. All amino acid residues except glycine have the L configuration.

min, 66 mL of hydrogen had been consumed (calcd 70 mL). After the reaction was complete, the catalyst was removed by filtration, and 2 equiv of 1 N KOH was added. The potassium salt of P-Leu-NH₂ (0.3 g) was crystallized from a waterethanol mixture to yield 0.1 g with NMR (D₂O) δ 3.7 (b, 1 H, NCHCO), 1.2 (m, 3 H, CH₂CH), 0.93 (d, 6 H, (CH₃)₂CH). Anal. Calcd for C₆H₁₄N₂O₄PK·H₂O: C, 27.05; H, 6.01; N, 10.52. Found: C, 27.26; H, 5.43; N, 10.67.

Dipeptide Benzyl Esters. The mixed anhydride method was used to synthesize Boc-Ala-Ala-OBzl (mp 67–68 °C), Boc-Gly-Ala-OBzl (oil), Boc-Ile-Ala-OBzl (mp 108–110 °C), Boc-Leu-Phe-OBzl (mp 98–100 °C), and Boc-Leu-Trp-OBzl (mp 127–130 °C). The corresponding formate salts were prepared by removal of the Boc group with 92% formic acid (Halpern & Nitecki, 1967). Then 1.2 equiv of 1.4 N HCl in dioxane was added to the residue. After 5 min, ether was added to solidify the hydrochloride salt in approximately 90% yield: H-Gly-Ala-OBzl·HCl (mp 205–209 °C), H-Ala-Ala-OBzl·HCl (mp 159–160 °C), H-Leu-Phe-OBzl·HCl (mp 163–164 °C), and H-Leu-Trp-OBzl·HCl (mp 146–149 °C).

Dibenzylphosphoryl Dipeptide Benzyl Esters. These were prepared by phosphorylation of the corresponding dipeptide benzyl ester hydrochlorides with (BzlO)₂POCl. The products were crystallized from methanol-ester-petroleum ether in 65-70% yield. (BzlO)₂PO-Gly-Ala-OBzl: mp 73-75 °C; NMR (CDCl₃) δ 7.4 (s, 15 H, Ph), 5.2 (s, 2 H, PhCH₂OCO), 5.1 (d, 4 H, PhCH₂OP), 4.6 (m, 1 H, NCHCO), 3.5 (d, 2 H, NCH₂CO), 1.3 (d, 3 H, CH₃CH). Anal. Calcd for C₂₆H₂₉N₂O₆P: C, 62.90; H, 5.85; N, 5.65. Found: C, 62.83; H, 5.88; N, 5.71. (BzlO)₂PO-Ala-Ala-OBzl: mp 102-103 °C; NMR (CDCl₃) δ 7.4 (s, 15 H, Ph), 5.2 (s, 2 H, PhCH₂OCO), 5.1 (d, 4 H, PhCH₂OP), 1.3 (d, 6 H, CH₃CH). Anal. Calcd for $C_{27}H_{31}N_2O_6P$: C, 63.53; H, 6.08; N, 5.49. Found: C, 63.34; H, 6.16; N, 5.57. (BzlO)₂PO-Ile-Ala-OBzl: mp 143-145 °C; NMR (CDCl₃) δ 5.2 (s, 2 H, PhCH₂OCO), 5.1 (m, 4 H, PhCH₂OP), 1.3 (d, 3 H, CH₃CH), 1.0 (d, 6 H, $(CH_3)_2$ CH). Anal. Calcd for $C_{30}H_{37}N_2O_6P$: C, 65.22; H, 6.70; N, 5.07. Found: C, 65.02; H, 6.78; N, 5.08. (BzlO)₂PO-Leu-Phe-OBzl: mp 100-101 °C; NMR (CDCl₃) δ 5.1 (s, 2 H, PhCH₂OCO), 5.0 (m, 4 H, PhCH₂OP), 3.0 (d, 2 H, PhCH₂CH), 0.8 (d, 6 H, (CH₃)₂CH). Anal. Calcd for C₃₆H₄₁N₂O₆P: C, 68.79; H, 6.53; N, 4.46. Found: C, 68.76; H, 6.56; N, 4.51. (BzlO)₂PO-Leu-Trp-OBzl: mp 120-122 °C; NMR (CDCl₃) δ 7.3 (m, 20 H, indole and Ph), 5.0 (s, 2 H, PhCH₂OCO), 4.9 (d, 4 H, PhCH₂OP), 3.2 (d, 2 H, CH_2 -indole), 0.8 (d, 6 H, (CH_3)₂CH). Anal. Calcd for C₃₈H₄₂N₃O₆P: C, 68.37; H, 6.30; N, 6.30. Found: C, 68.21; H, 6.36; N, 6.31.

Phosphoryl Dipeptides. Potassium salts were prepared by hydrogenolysis (atmospheric pressure) of the corresponding benzyl ester followed by treatment with 1 N KOH. The following compounds were prepared. Tripotassium salt of P-Ala-Ala-OH: NMR (D₂O) δ 4.2–3.6 (m, 1 H, NCHCO), 1.4 (d, 6 H, CH_3CH). Anal. Calcd for $C_6H_{10}N_2O_6PK_3 \cdot 3H_2O$: C, 17.65; H, 3.92; N, 6.86. Found: C, 17.26; H, 3.37; N, 6.68. Tripotassium salt of P-Ile-Ala-OH: NMR (D_2O) δ 1.4 (d, 3 H, CH_3CH), 1.0 (d, 6 H, $(CH_3)_2CH$). Anal. Calcd for C₉H₁₆N₂O₆PK₃·3H₂O: C, 23.98; H, 4.89; N, 6.22. Found: C, 24.46; H, 5.31; N, 6.36. Tripotassium salt of P-Leu-Phe-OH: NMR (D_2O) δ 7.3 (s, 5 H, Ph), 3.1 (d, 2 H, PhCH₂CH), 0.8 (d, 6 H, $(CH_3)_2$ CH). Anal. Calcd for C₁₅H₂₀N₂O₆PK₃: C, 38.38; H, 4.23; N, 5.92. Found: C, 38.38; H, 4.31; N, 5.94. Dipotassium salt of P-Leu-Trp-OH: NMR (D_2O) δ 7.3 (m, 5 H, indole), 3.4 (d, 2 H, CH₂-indole), 0.95, 0.85 (d, 6 H, $(CH_3)_2CH$). Anal. Calcd for

 $C_{17}H_{22}N_3O_6PK_2H_2O$: C, 41.55; H, 4.89; N, 8.55. Found: C, 41.57; H, 5.18; N, 8.56.

Phosphorylphenylalanine (P-Phe-OH). Dibenzylphosphorylphenylalanine benzyl ester was prepared by reaction of (BzIO)₂POCl with H-Phe-OBzl and had mp 88–90 °C; NMR (CDCl₃) δ 7.3 (b, 20 H, Ph), 5.0 (s, 2 H, PhCH₂OCO), 4.9 (m, 4 H, PhCH₂OP), 2.9 (d, 2 H, PhCH₂CH). Anal. Calcd for C₃₀H₃₀NO₅P: C, 69.90; H, 5.82; N, 2.73. Found: C, 69.85; H, 5.85; N, 2.72. The tripotassium salt of phosphorylphenylalanine was prepared by hydrogenolysis (atmospheric pressure) and had NMR (D₂O) δ 7.4 (s, 5 H, Ph), 3.1 (d, 2 H, PhCH₂CH). Anal. Calcd for C₉H₉N₁O₅PK₃·H₂O: C, 28.62; H, 2.92; N, 3.71. Found: C, 29.01; H, 3.25; N, 3.75.

*O-Methyl-O-phenylphosphorylleucinamide. O-*Methyl *O*-phenyl phosphochloridate was prepared by the procedure of Hamer & Tack (1974) and was used to phosphorylate leucinamide. The reaction mixture was purified by chromatography on a silica gel column. The eluted product was crystallized from ethyl acetate (38% yield) and had mp 128–130 °C; NMR (CDCl₃) δ 7.2 (s, 5 H, Ph), 3.7 (d, 3 H, CH₃OP, $J_{P-CH} = 10$ Hz), 0.8 (d, 6 H, (CH₃)₂CH). Anal. Calcd for C₁₃H₂₁N₂O₄P: C, 52.00; H, 7.00; N, 9.33. Found: C, 51.86; H, 7.06; N, 9.29.

O-Methylphosphorylleucinamide. O-Methyl-O-phenylphosphorylleucinamide (0.3 g) was dissolved in 50 mL of 0.2 M KOH and the solution was stirred at 25 °C for 3 h. Then the solution was titrated to pH 7 with 2.5 N HCl and washed with ethyl acetate to remove phenol. Potassium chloride was precipitated by addition of methanol and filtered. Acetone was then added to precipitate the product. The final product was crystallized from methanol and acetone in 35% yield and had NMR (D₂O) δ 3.5 (d, 3 H, CH₃OP), 0.9 (d, 6 H, (CH₃)₂CH). Anal. Calcd for C₇H₁₆N₂O₄PK·H₂O: C, 29.99; H, 6.43; N, 10.00. Found: C, 30.14; H, 5.89; N, 10.02.

N-Ethylphosphoramidate. 0,0-Dibenzyl *N*-ethylphosphoramidate was synthesized by reaction of $(BzlO)_2POCl$ and ethylamine in ether solution. The product was purified by silica gel chromatography and had NMR $(CDCl_3) \delta$ 7.4 (s, 10 H, Ph), 5.1 (d, 4 H, PhCH₂OP), 3.5 (b, 1 H, NH), 3.0 (m, 2 H, CH₃CH₂N), 1.1 (t, 3 H, CH₃CH₂N). The tripotassium salt of *N*-ethylphosphoramidate was synthesized by hydrogenolysis and treatment with 1 N KOH and had NMR $(D_2O) \delta$ 3.0 (m, 2 H, CH₃CH₂N), 1.3 (t, 3 H, CH₃CH₂N); R_f 0.3 (2-propanol:H₂O, 7:3).

Thermolysin Kinetic Studies. Most K_{I} values with thermolysin were determined in a 0.1 M Tris-HCl, 10 mM CaCl₂, pH 7.2 buffer with FA-Gly-Leu-NH₂ as a substrate (Walsh et al., 1975; Feder, 1968) by using a Beckman Model 25 spectrophotometer. Dixon and Henderson plots were used for the calculation of $K_{\rm I}$ values. A new fluorogenic substrate Abz-Ala-Gly-Leu-Ala-Nba (N. Nishino and J. C. Powers, unpublished experiments) was also employed with phosphoramidon and P-Leu-Trp-OH. A mixture of inhibitor (2 mL) and thermolysin (50 μ L) was incubated at 25 °C for 15 min in 0.05 M Tris-HCl, 2.5 mM CaCl₂, pH 7.2 buffer in a cuvette. Fifty microliters of substrate solution in dimethylformamide was then added and the increase in fluorescence was measured with a Perkin-Elmer 204-S fluorescence spectrophotometer at ex 340 and em 415 nm. Dixon and Henderson plots with two substrate concentrations (0.1 and 0.05 mM) and various inhibitor concentrations (10-80 nM) were used to calculate $K_{\rm I}$ values. The thermolysin concentration was determined from the extinction coefficient $(E_{280}^{1\%})$ = 17.6). The concentration of phosphoramidon was calculated

from the extinction coefficient ($E_{282}^{\text{MeOH}} = 5000$) (Umezawa et al., 1972).

Rate of Association of Inhibitors with Thermolysin. To 2 mL of a phosphoramidon (440 nM) or P-Leu-Trp-OH (160 nM) solution in 0.05 M Tris HCl, 2.5 mM CaCl₂, pH 7.2, was added 50 μ L of a thermolysin (3.5 nM) solution in the same buffer at 25 °C. After incubation for the appropriate interval, 50 μ L of 4 mM substrate (Abz-Ala-Gly-Leu-Ala-NBa) in dimethylformamide was added. The final concentrations were 0.88 nM thermolysin and 0.1 mM substrate. The initial increase in fluorescence was followed as above.

Dissociation of the Thermolysin-Phosphoramidon Complex. In a dialysis experiment, a mixture of $3.5 \ \mu$ M thermolysin and $4 \ \mu$ M phosphoramidon in 0.5 mL of a pH 7.2 buffer was dialyzed overnight at 4 or 25 °C against 500 mL of 0.05 M Tris, 2.5 mM CaCl₂, pH 7.2 buffer. Five microliters of each solution was assayed fluorometrically as described above. Residual activity before dialysis was 3%; after dialysis, 90% activity was obtained.

In another experiment, an affinity resin (HONH- $COCH(CH_2C_6H_5)CO-Ala-Gly-NH(CH_2)_3$ -agarose) with a thermolysin-specific ligand was utilized (Nishino & Powers, 1978, and unpublished results). Thermolysin and phosphoramidon were incubated at concentrations of 3.5 μ M and 4 μ M (1:1.14), respectively, in 0.05 M Tris-HCl, 2.5 mM CaCl₂, pH 7.2 buffer at 25 °C for 1 h. Approximately 3% residual activity was observed. One hundred microliters of the mixture was applied to the hydroxamic acid affinity column (4 mL of HONHCOCH($CH_2C_6H_5$)CO-Ala-Gly-NH(CH_2)₃-agarose). The column was washed with 0.1 M Tris-HCl, 10 mM CaCl₂, pH 7.2 buffer. A fairly slow flow rate (6 mL/h) was chosen for the first five 2-mL fractions since kinetic studies indicated that the complex dissociated slowly. After thorough washing at a flow rate of 20 mL/h, the thermolysin bound on the column was eluted 0.1 M Tris-HCl, 0.1 M CaCl₂, pH 9.0. Fifty microliters of each 2-mL fraction was assayed for thermolysin activity by using Abz-Ala-Gly-Leu-Nba as a substrate (0.1 mM). The first five fractions which still contained enzyme activity were combined and reapplied to the affinity column.

Carboxypeptidase A Kinetic Studies. The Z-Gly-Phe-OH assay of Petra (1970) was used. The enzyme concentration was determined spectrophotometrically $(E_{278}^{0.4\%} = 0.75)$.

Results

Inhibitors of Thermolysin. Several N-phosphorylated amino acids and dipeptides were shown to inhibit thermolysin. The inhibition data of thermolysin is summarized in Table I. P-Leu-NH₂ shows strong inhibition with a K_1 of 1.3 μ M, while unphosphorylated H-Leu-NH2 is a very weak inhibitor (Feder et al., 1974) and N-ethylphosphoramide does not inhibit the enzyme at 3 mM. The three dipeptides, P-Ile-Ala-OH, P-Leu-Phe-OH, and P-Leu-Trp-OH, inhibit the enzyme better than P-Leu-NH₂. P-Ala-Ala-OH has a larger K_I value than the other N-phosphorylated dipeptides and P-Leu-NH₂. The main difference is the amino acid adjacent to the phosphoryl group; compounds with hydrophobic amino acids at the $P_1^{\prime 2}$ position show smaller K_1 values. P-Phe-OH which has a free carboxyl group also inhibits thermolysin, but fivefold weaker than P-Leu-NH₂. O-Methyl-O-phenylphosphoryl-Leu-NH₂ does not inhibit thermolysin at 3 mM, and the K_1 value of

Table I: Thermolysin Inhibitors

compd	concn (µM)	$K_{\rm I}{}^a$ ($\mu { m M}$)
H-Leu-NH, ^b	4900-29 000	12000
P-NH-Et	3100	no inhibn
P-Leu-NH ₂	0.62-3.10	1.3
O Č		
MeO-P-Leu-NH,	50-200	150
OK Í		
0		
Ĭ		
MeO-P-Leu-NH ₂	3100	no inhibn
OC H.		
P-Phe-OH	27-106	73
P-Ala-Ala-OH	46-184	88
P-Ile-Ala-OH	0.17-1.0	0.36
P-Leu-Phe-OH	0.044 - 0.11	0.019
P-Leu-Trp-OH	0.010-0.040	0.015^{c}
phosphoramidon	0.03-0.12	0.034
	0.02-0.08	0.032 ^c

^a All kinetic studies were performed at pH 7.2, 25 °C, FA-Gly-Leu-NH₂, 0.95-3.3 mM. Thermolysin, 0.03-0.56 μ M. ^b Feder et al. (1974). ^c Abz-Ala-Gly-Leu-Ala-Nba was used as a substrate in place of FA-Gly-Leu-NH₂, 0.05 and 0.1 mM. Thermolysin, 0.88 nM.

O-methylphosphoryl-Leu-NH₂ is approximately 100-fold larger than the $K_{\rm I}$ of P-Leu-NH₂. The $K_{\rm I}$ value of phosphoramidon which we obtained compares quite favorably with those previously reported (Komiyama et al., 1975a,b). However, the value reported for P-Leu-Trp-OH is considerably lower (2–6 nM) than that which we obtained (15 nM). This discrepancy may be due to the fact that Komiyama et al. (1975a) did not isolate and purify P-Leu-Trp-OH, but prepared it in situ by hydrolysis.

Kinetics of the Inhibition. Owing to the low solubility (ca. 3 mM) of the substrate FA-Gly-Leu-NH₂ and its high K_M value (ca. 30 mM), the inhibitors were examined under pseudo-first-order conditions. When [S] $\ll K_M$ competitive inhibition is described by the rate equation

$$d[p]/dt = k_{cat}[E][S]/K_{M}[1 + ([I]/K_{i})]$$

one cannot distinguish competitive from noncompetitive inhibitors because both yield the same rate expression (Feder et al., 1974).

A new fluorogenic thermolysin substrate Abz-Ala-Gly-Leu-Ala-Nba was synthesized (N. Nishino and J. C. Powers, unpublished experiments) to determine the mode of inhibition of some of the better inhibitors. This substrate is cleaved at the Gly-Leu bond with a resultant increase in fluorescence due to separation of the fluorescent group (Abz, 2-aminobenzoyl) from the quenching group (Nba, 4-nitrobenzylamide). The $K_{\rm M}$ and $k_{\rm cat}$ for this substrate in a 0.05 M Tris-HCl, 2.5 mM CaCl₂ buffer containing 2.5% dimethylformamide at 25 °C were respectively 0.14 mM and 283 s⁻¹. Under the conditions of the Abz-Ala-Gly-Leu-Ala-Nba assay, [S] was in the same range as the $K_{\rm M}$, and both phosphoramidon and P-Leu-Trp-OH were shown to be competitive inhibitors. The K_1 values obtained with phosphoramidon by using FA-Gly-Leu-NH₂ and Abz-Ala-Gly-Leu-Ala-Nba were essentially identical.

The K_1 values for the poorly bound inhibitors ($K_1 > ca. 1 \mu M$) were determined by Dixon plots (1/v vs. [I]) with the K_1 values being obtained from the [I] axis intercept. In the case of tightly bound inhibitors, a substantial amount of inhibitor may be complexed with enzyme in the form of the E-I complex. Standard Dixon plots which are based on the assumption that [I] = [I]_{total} cannot be used when [E] is similar

² The nomenclature used for the individual amino acid residues $(P_1', P_2', \text{ etc.})$ of an inhibitor and the subsites $(S_1', \text{ etc.})$ of the enzyme is that of Schechter & Berger (1967).



FIGURE 1: Rate of association of phosphoramidon (solid circles) and P-Leu-Trp-OH (open circles) with thermolysin (0.88 nM) at pH 7.2. The concentrations of phosphoramidon and P-Leu-Trp-OH were 400 and 160 nM, respectively. The activity was measured by adding the substrate Abz-Ala-Gly-Leu-Ala-Nba to the incubation mixture.

to [I] (Segel, 1975). For inhibitors with $K_I > \text{ca. 1 } \mu M$, and [I] \simeq [E], the K_I values were determined with Henderson plots (Henderson, 1972) with the slope being equal to K_I . Nearly identical values were obtained with Dixon plots.

Association of the Inhibitors with Thermolysin. The K_1 values of ordinary reversible inhibitors can be easily estimated from kinetics due to the fast equilibrium formation of the E-I complex. During the course of the kinetic work, we observed that this was not the case with phosphoramidon. We found that the rate of hydrolysis of FA-Gly-Leu-NH₂ decreased very fast initially in the presence of phosphoramidon. If the enzyme and inhibitor were first preincubated, no substrate hydrolysis occurred. These results indicate that the binding of phosphoramidon to thermolysin does not initially reach equilibrium and is consistent with the following kinetic scheme:

$$E + I \xrightarrow[k_{-1}]{k_{-1}} E \cdot I \quad k_1 \text{ slow}$$

In this scheme, the formation of the E-I complex is not fast and, thus, the rate of FA-Gly-Leu-NH₂ hydrolysis will decrease as the E-I complex is formed in the assay mixture.

The time course for the association of phosphoramidon and P-Leu-Trp-OH is shown in Figure 1. Under the conditions of the experiment, equilibrium with P-Leu-Trp-OH is reached in 3-4 min, while phosphoramidon takes over twice as long. This behavior is unique to phosphoramidon and P-Leu-Trp-OH and was not observed with any of the other inhibitors. It also seems to be characteristic of thermolysin. Pseudomonas aeruginosa elastase, a metalloprotease with a substrate specificity similar to that of thermolysin, is also inhibited by phosphoramidon with a $K_{\rm I}$ value of 55 nM. In contrast to thermolysin, the association rate of phosphoramidon with the P. aeruginosa elastase was found to be fast (N. Nishino and J. C. Powers, unpublished experiments). The $K_{\rm I}$ values for phosphoramidon and P-Leu-Trp-OH were measured from enzyme-inhibitor solutions which had been allowed to incubate for 15 min to allow equilibration to be complete.

Dissociation of the Phosphoramidon-Thermolysin Complex. We next studied the dissociation of the thermolysinphosphoramidon complex to demonstrate the active enzyme could be recovered. Dialysis of a solution of thermolysin and phosphoramidon (3% residual activity) overnight against a pH

Table II: Inhibitors for Carboxypeptidase A

compd	concn (µM)	type of inhibn	$K_{\rm I}{}^a(\mu{\rm M})$
P-Phe-OH	1.6-4.2	competitive	5.0
P-Leu-NH ₂	31-78	competitive	160
	1.600		
MeO-P-Leu-NH ₂	1600	no inhibn	
OC ₆ H ₅			
P-Leu-Trp-OH ^b	16-64	competitive	54
P-Ala-Ala-OH	150-430	competitive	700
P-NH-C ₂ H ₅	110-430	competitive	28 0
Q			
BzlO-P-Gly-Ala-OH	800	no inhibn	
ÓBzl			
phosphate	3000-30 000	no inhibn	

^a All kinetic studies were performed at pH 7.5, 25 °C, Z-Gly-Phe-OH, 0.5-2 mM; [E], 0.15-0.32 μ M. ^b P-Leu-Trp-OH is not a substrate.

7.2 buffer led to the recovery of over 90% of the original thermolysin. A similar result was obtained by using P-Phe-OH instead of phosphoramidon.

In a separate experiment, an affinity resin (HONH- $COCH(CH_2C_6H_5)CO-Ala-Gly-NH(CH_2)_3$ -agarose) with a thermolysin specific ligand was utilized (Nishino & Powers, 1978, and unpublished results). This resin completely retains active thermolysin at pH 7.2. The enzyme can then be eluted with a pH 9.0 buffer or by the use of thermolysin inhibitors in the eluting buffer. When the phosphoramidon-thermolysin complex was applied to the affinity resin, 66% of the original thermolysin activity was retained and could be eluted with a pH 9.0 buffer. The remainder of the thermolysin activity (31%) was washed through the column by the pH 7.2 buffer probably due to the presence of phosphoramidon. The $K_{\rm I}$ for phosphoramidon is ca. tenfold lower than that of the hydroxamic acid inhibitor which was used as the ligand on the column (Nishino & Powers, 1978). The thermolysin, which was not retained by the column during the initial experiment, was reapplied to the column and found to be mainly retained the second time. Thus, the phosphoramidon which was present in the initial solution of the phosphoramidon-thermolysin complex was responsible for eluting part of the thermolysin off the hydroxamic acid column.

The dialysis and affinity chromatography experiments demonstrate that phosphoramidon can be dissociated from thermolysin. However, there is one indication that the rate is not fast. When substrate (Abz-Ala-Gly-Leu-Ala-Nb) is added to a solution of thermolysin and phosphoramidon, the rate of substrate hydrolysis accelerates for 1-2 min before becoming a straight line. This could be explained if the rate of dissociation of the E-I complex was slow and the steady state required 1-2 min to reach. A similar behavior was observed with P-Leu-Trp-OH, but of much shorter duration. No attempt was made to measure the dissociation rates.

Inhibitors of Carboxypeptidase A. The inhibition data are summarized in Table II. P-Phe-OH is the best carboxypeptidase A inhibitor in this series. Although P-Leu-NH₂ does not have a free carboxyl group, it is still a moderate inhibitor of the enzyme. The dipeptides, P-Leu-Trp-OH and P-Ala-Ala-OH, also show inhibition, but with 10- and 140-fold higher K_I values, respectively, than P-Phe-OH. The K_I value of P-Leu-Trp-OH is ca. 13-fold smaller than P-Ala-Ala-OH. Since P-Leu-Trp-OH has a carboxyl-terminal tryptophan residue, it was checked spectrophotometrically for hydrolysis by carboxypeptidase A. Incubation of a 1 mM solution of P-Leu-Trp-OH and 0.24 μ M of enzyme at room temperature for 1 h results in no OD change at 224 nm. The cleavage of peptides such as Z-Gly-Phe-OH by carboxypeptidase is commonly measured by this method and used to determine the peptidase activity of the enzyme (Petra, 1970). Thus P-Leu-Trp-OH appears to be stable to carboxypeptidase hydrolysis. The alkylphosphoramidate P-NH-Et has a K_1 value similar to that of P-Leu-NH₂. Both phosphate and pyrophosphate do not inhibit the enzyme, as has been also shown in previous work (Neurath & DeMaria, 1950). Both *O*-methyl-*O*-phenylphosphoryl-Leu-NH₂ and dibenzylphosphoryl-Gly-Ala-OH are not inhibitors at 1 mM. The requirement for a free phosphoryl group is thus indicated.

Discussion

The phosphoramidate, phosphoramidon [N-(α -L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan], was isolated by Umezawa and his co-workers from Actinomycetes culture filtrates and shown to be a powerful competitive inhibitor of thermolysin (Komiyama et al., 1975a). The non-rhamnose-containing analogues P-Leu-Trp-OH and P-Leu-His-OH were also inhibitors with the former binding more tightly to phosphoramidon. Phosphoramidon inhibits other bacterial neutral metalloendopeptidases related to thermolysin and has been used as a ligand for affinity chromatography (Komiyama et al., 1975b). Another synthetic phosphoramidate ligand (O-phenylphosphoryl-Phe-Phe-) has been used to purify the zinc neutral endoprotease from Bacillus cereus (Holmquist, 1977). The goal of this research was to elucidate the mode of binding of phosphoramidates to thermolysin, to uncover the important structural features of the inhibitor, and to extend this class of inhibitors to other metalloproteases.

Due to the preference of thermolysin for substrates with a P_1 leucyl, isoleucyl, or phenylalanyl residue, we expected the interaction between the leucyl side chain of phosphoramidon and the S_1 binding subsite of thermolysin to make a significant contribution to the total binding energy. The second important interaction was expected to be the coordination of one oxygen of the phosphoryl group of the inhibitors with the active site zinc of the enzyme. This interaction is analogous to interaction of the carbonyl oxygen of the scissile peptide bond of a substrate with the zinc atom of thermolysin (Kester & Matthews, 1977a). In addition, the phosphoramidate functional group has the same geometry as the tetrahedral transition state involved in peptide bond hydrolysis and, thus, its binding to the enzyme should be stabilized. Therefore, we synthesized P-Leu-NH₂ with the expectation that this simple phosphoramidate would contain most of the structural features of phosphoramidon necessary for inhibition of thermolysin.

The simple phosphoramidate P-Leu-NH₂ was indeed a significant inhibitor of thermolysin with a K_1 value of 1.3 μ M. Neither H-Leu-NH₂ nor the simple phosphoramidate P-NH-Et were significant inhibitors, indicating the necessity for a cooperative effect of both the phosphoryl group and the hydrophobic side chain of the amino acid at the P_1 ' site. The inhibitor is bound at least 1000-fold more tightly to thermolysin than most substrates which have $K_{\rm M}$ values in the 2-20 mM range (Morihara & Tsuzuki, 1970). A reasonable lower estimate for the K_M of a substrate with the same sequence as phosphoramidon is ca. 3 mM. The K_M for Z-Gly-Leu-Phe-OH is 2.4 mM (Morihara & Tsuzuki, 1970) and, although the difference between Phe and Trp is probably insignificant (as is shown with the dipeptide inhibitors), the Z-glycyl moiety probably makes a substantial contribution to the binding. In addition, Komiyama et al. (1975a,b) report that the $K_{\rm M}$ for Z-Gly-Leu-Trp-OH is one order of magnitude lower than



FIGURE 2: A schematic drawing of the interactions observed in the phosphoramidon-thermolysin complex.

Z-Gly-Leu-NH₂ which would place it in the 2–3 mM range. But by using a value of 3 mM for a substrate, the free energy of binding of a substrate, P-Leu-NH₂, and phosphoramidon are respectively 3.6, 8.0, and 10 kcal/mol. Of the differences in free energy of binding between phosphoramidon and a substrate, over 65% is accounted for by the structural features in P-Leu-NH₂. Interactions with the Trp residue and the rhamnose ring must account for the remainder.

N-Phosphoryl dipeptides inhibit the enzyme up to 85-fold better than P-Leu-NH₂, indicating that interaction between the S₂' subsite of the enzyme and the P₂' residue of the inhibitor can contribute further to the binding energy. However, a hydrophobic P₁' amino acid is still required since P-Ala-Ala-OH binds 240-fold less tightly than P-Ile-Ala-OH. A P₂' aromatic residue also contributes to the binding since P-Leu-Trp-OH has a K₁ which is over 20 times lower than that of P-Ile-Ala-OH. However, little difference is observed between a P₂' Trp or Phe. The *N*-phosphoryl tripeptide P-Ile-Ala-Gly-OEt (K₁ = 0.31 μ M) and tetrapeptide P-Ile-Ala-Gly-Gln-NH₂ (K₁ = 0.24 μ M) inhibit thermolysin only as well as P-Ile-Ala-OH, indicating that no significant interactions take place beyond the S₂' subsite.

The structure of the phosphoramidon-thermolysin complex has been determined by X-ray crystallography, and Figure 2 schematically represents the interactions observed in the vicinity of the phosphoramidate functional group (Weaver et al., 1977). In the complex, the side chain of the leucyl residue fits into the hydrophobic S_1' pocket and the NH of the phosphoramidate is hydrogen bonded to the peptide carbonyl group of Ala-113. The P=O oxygen is coordinated to the active site zinc atom and the P-OH oxygen is hydrogen bonded both to Glu-143 and a water molecule which is in turn hydrogen bonded with the NH of the peptide bond of Trp-115. There are additional interactions involving the rhamnose and tryptophan rings.

There is one difficulty with this model which cannot be clarified by crystallography. The pK's of simple phosphoramidate monoesters such as CH₃OPO(OH)NH₂ and CH₃OPO(OH)NH-C₆H₁₁ are 2.5-3.1 (Oney & Caplow, 1967), and phosphoramidon should be ionized at neutral pH. The negatively charged oxygen thus formed would be expected to coordinate to the electropositive zinc atom. Evidence for the requirement of a negatively charged oxygen for coordination is found in the observation that O-methyl-O-phenylphosphoryl-Leu-NH₂, which lacks this structural feature, is not an inhibitor. Although this could be due to steric problems, there seems to be no obvious reason why the phenyl group of O-methyl-O-phenylphosphoryl-Leu-NH2 could not have replaced the rhamnose ring in Figure 2, while the methoxy group replaced the phosphorus OH and the water molecule. Additional evidence for the requirement of a negative charge for coordination can be found in the observation that peptides with



FIGURE 3: A schematic drawing of the proposed interactions between $P-Leu-NH_2$ and thermolysin.

the sulfamic acid functional group ($^{-}OSO_2NHR$) are inhibitors of thermolysin, while those with sulfonamides (CH₃SO₂NHR) are not (N. Nishino and J. C. Powers, unpublished observations).

If the negatively charged oxygen of the P–O bond were coordinating to the zinc atom, then the P==O bond would then be hydrogen bonded to the water molecule and Glu-143 as shown in Figure 3. This would require Glu-143 to be protonated. It should be noted that Glu-143 should be protonated even in the model represented in Figure 2 since the pK's of simple carboxylic acids (ca. 4.8) are slightly higher than those of monoesters of phosphoramidates (2.5–3.1). Such a proton which was interacting with two oxygen atoms (Figure 3) could indeed have quite a high pK. Examples of such stabilization can be found in the second pK of 1,2-*cis*-cyclopropanedicarboxylic acid (pK = 6.5) and maleic acid (6.2), and in the pK's of (HO)₂POCH₂PO(OH)₂ (<2, 2.6, 6.9, and 10.3).

In the two examples which we investigated, monoesters of phosphoramidates were weaker inhibitors than phosphoramidates. MeO-PO(OH)-Leu-NH₂ was 115-fold poorer than P-Leu-NH₂, and phosphoramidon was bound only half as tightly as P-Leu-Trp-OH. Since the rhamnose ring of phosphoramidon makes several contacts with the enzyme (Weaver et al., 1977) and probably makes some contribution to the free energy of binding, a greater difference would be expected between P-Leu-Trp-OH and its monomethyl ester than is observed when the K_1 values of P-Leu-Trp-OH and phosphoramidon are compared.

We propose that the binding of unsubstituted phosphoramidates to thermolysin is stabilized by additional interactions with the enzyme. In particular, an electrostatic interaction between the imidazole of His-231 and the negatively charged P-O bond of the phosphoramidate functional group, and a hydrogen bond with the hydroxyl group of Tyr-153 as shown in Figure 3, appears to be a likely candidate. At the pH of our studies, phosphoramidates would exist as monoanions. Previous studies have shown that these monoanions are zwitterionic as shown in Figure 3 (Hobbs et al., 1953; Benkovic & Sampson, 1972). Protonation of the nitrogen should not affect the hydrogen bond with Ala-113. But the monoanion would have an additional negatively charged oxygen as compared with monoesters such as phosphoramidon. This would result in a significant negative charge on all three of the phosphorus oxygen atoms (Figure 3 represents only one of the possible resonance structures).

Using the published coordinates (Weaver et al., 1977; Matthews et al., 1974), we have calculated the distance separating the phenolic oxygen of Tyr-153 and the oxygen of the P–O bond to be 2.2 Å. This oxygen is bonded to the rhamnose ring in phosphoramidon. Allowing for some inaccuracy in the coordinates, this is certainly reasonable to allow for formation of a hydroben bond as shown in Figure 3. The separation between this oxygen and N^{ϵ} of His-231 is 3.7 Å, much too far for a direct hydrogen bond. However, His-231 is close enough for an electrostatic interaction probably via an intervening water molecule as shown in Figure 3. We believe that one or both of these interactions account for the lower $K_{\rm I}$ values of phosphoramidates compared with their monesters.

It would also be possible to explain the differences in K_1 values between phosphoramidates and their monalkyl esters if the former were for some reason better ligands for the zinc atom in thermolysin. This seems to be an unlikely explanation since the pK's of phosphoramidates (pK of N-butylphosphoramidate = 2.9) and monoalkyl esters (pK = 2.5-3.1) are essentially the same (Benkovic & Sampson, 1971; Oney & Caplow, 1967). Taking this as a measure of how the two interact with one Lewis acid (a proton), then little difference in their ability to coordinate to another Lewis acid (the active site zinc atom) would be expected.

The K_1 value of 0.034 μ M for phosphoramidon determined by using the FA-Gly-Leu-NH₂ assay compares favorably with those determined by Umezawa and his colleagues by kinetics $(0.028 \ \mu M)$ or difference spectroscopy $(0.088 \ \mu M)$ (Komiyama et al., 1975a,b). However, use of the FA-Gly-Leu-NH₂ and Abz-Ala-Gly-Leu-Ala-Nba assays and continuous recording of product formation showed that initially phosphoramidon was not binding to thermolysin. In fact 5-15 min are required for complex formation (Figure 1). The casein and Z-Gly-Leu-NH₂ assays used by Komiyama et al. (1975a,b) determine product only after 30 or 20 min of reaction, respectively, and would not detect the time dependence of the binding. Except for phosphoramidon and P-Leu-Trp-OH, no other phosphoramidate showed this kinetic behavior. Our results indicate that the rate of formation of the E-I complex with these two inhibitors is slow. This phenomenon commonly occurs in the formation of complexes of trypsin with soybean trypsin inhibitor and pancreatic trypsin inhibitor (Green, 1953; Laskowski & Sealock, 1971).

Thermolysin undergoes several localized conformational changes on binding phosphoramidon (Weaver et al., 1977). One of these involves the side chain of Asn-112 which rotates to interact with the backbone of the P_2' Trp residue of phosphoramidon. Possibly a slow conformational change in this region of thermolysin is required before P-Leu-Trp-OH and phosphoramidon can bind. This behavior seems to be limited to thermolysin and was not observed with the related metalloprotease *Pseudomonas aeruginosa* elastase which is also inhibited by phosphoramidon (Morihara & Tsuzuki, 1978). With elastase the rate of association is rapid ($K_I = 0.055 \ \mu$ M; N. Nishino and J. C. Powers, unpublished experiments).

Once we felt that we understood the binding of phosphoramidates to thermolysin, we decided to see if this type of inhibitor could be applied to another metalloprotease. Carboxypeptidase A, a metalloendopeptidase with different substrate specificity, seemed like an ideal choice. A recent comparison of the crystal structures of carboxypeptidase A and thermolysin has shown that both enzymes have similar structural features in their active sites and share a common mechanism for peptide bond hydrolysis (Kester & Matthews, 1977b). Since the major recognitions between carboxypeptidase A and a substrate are interactions between the side chain of the P_1' residue and a hydrophobic S_1' binding pocket, and between the carboxylate of a substrate and Arg-145, we felt that P-Phe-OH would make an excellent inhibitor. This was in fact the best phosphoramidate inhibitor of carboxypeptidase A which we investigated ($K_i = 5.0 \ \mu M$). Simple phosphoramidates (P-NH-Et, $K_{\rm I}$ = 280 μ M) or those lacking the carboxyl group (P-Leu-NH₂, $K_{\rm I} = 160 \ \mu M$) were much poorer. Dipeptides such as P-Leu-Trp-OH and P-Ala-Ala-OH still inhibited the enzyme, probably because a hydrogenbonding interaction between the peptide bond of the inhibitors and Arg-145 replaces the ionic interaction between the carboxylate of P-Phe-OH and Arg-145. A similar interaction involving Arg-203 is seen in the binding of dipeptides to thermolysin (Kester & Matthews, 1977a) and explains why P-Phe-OH is a reasonable thermolysin inhibitor. Blocking the phosphoryl oxygens eliminated the inhibition. The possibility that the dipeptide phosphoramidates were hydrolyzed by carboxypeptidase A was checked with P-Leu-Trp-OH. No hydrolysis could be detected after incubation for 1 h. Thus, the key structural features for a carboxypeptidase inhibitor are the phosphoryl group, an aromatic or hydrophobic P_1 residue and ideally a P_1' carboxylate.

In conclusion, phosphoramidate derivatives of amino acids and peptides appear to be a general class of reversible inhibitors for metalloproteases. Individual phosphoramidates have now been shown to inhibit thermolysin, two related metalloendopeptidases (Komiyama et al., 1975b), carboxypeptidase A, *P. aeruginosa* elastase (Morhara & Tsuzuki, 1978), and a zinc neutral endoprotease from *Bacillus cereus* (Holmquist, 1977). The inhibition of a number of separate zinc metalloproteases by phosphoramidates containing differing recognition features provides further evidence for the essential similarity of the catalytic sites of most metalloproteases. The substrate recognition site(s) in contrast display considerable diversity. Finally, phosphoramidate derivatives of the appropriate amino acids or peptides should be useful as reversible inhibitors or affinity ligands for other metalloproteases.

Acknowledgments

The authors are grateful to Dr. Brian Matthews for discussions of the structure of the phosphoramidon-thermolysin complex prior to publication. We also thank Dr. T. Aoyagi, Dr. W. Troll, and Dr. K. Walsh for providing samples of phosphoramidon.

References

- Atherton, F. R., Howard, H. T., & Todd, A. R. (1948) J. Chem. Soc., 1106.
- Benkovic, S., & Sampson, E. (1971) J. Am. Chem. Soc. 93, 4009.
- Byers, L. D., & Wolfenden, R. (1972) J. Biol. Chem. 247, 606.
- Byers, L. D., & Wolfenden, R. (1973) Biochemistry 12, 2070.
- Feder, J. (1968) Biochem. Biophys. Res. Commun. 32, 326.
- Feder, J., & Schuck, J. M. (1970) Biochemistry 9, 2784.

- Feder, J., Brougham, L., & Wildi, B. (1974) *Biochemistry* 13, 1186.
- Green, N. M. (1953) J. Biol. Chem. 205, 535.
- Halpern, B., & Nitecki, D. (1967) Tetrahedron Lett. 31, 3031.
- Hamer, N. K., & Tack, R. D. (1974) J. Chem. Soc., Perkin Trans. 2, 1184.
- Henderson, P. J. F. (1972) Biochem. J. 127, 321.
- Hobbs, E., Corbridge, D. E. C., & Raistrick, B. (1953) Acta Crystallogr. 6, 621.
- Holmquist, B. (1977) Biochemistry 16, 4591.
- Kester, W. R., & Matthews, B. W. (1977a) *Biochemistry 16*, 2506.
- Kester, W. R., & Matthews, B. W. (1977b) J. Biol. Chem. 252, 7704.
- Kitz, R., & Wilson, I. B. (1962) J. Biol. Chem. 237, 3245.
- Komiyama, T., Suda, H., Aoyagi, T., Takeucki, T., Umezawa, H., Fujimoto, K., & Umezawa, S. (1975a) Arch. Biochem. Biophys. 171, 727.
- Komiyama, T., Aoyagi, T., Takeuch, T., & Umezawa, H. (1975b) Biochem. Biophys. Res. Commun. 65, 352.
- Laskowski, M., Jr., & Sealock, R. W. (1971) Enzyme, 3rd Ed. 3, 375.
- Li, S. (1952) J. Am. Chem. Soc. 74, 5959.
- Li, S., & Eakin, R. E. (1955) J. Am. Chem. Soc. 77, 1866.
- Matthews, B. W., Weaver, L. H., & Kester, W. R. (1974) J. Biol. Chem. 249, 8030.
- Morihara, K. (1967) Biochem. Biophys. Res. Commun. 26, 656.
- Morihara, K., & Oka, T. (1968) Biochem. Biophys. Res. Commun. 30, 625.
- Morihara, K., & Tsuzuki, H. (1970) Eur. J. Biochem. 15, 374.
- Morihara, K., & Tsuzuki, H. (1978) Jpn. J. Exp. Med. 48, 81.
- Morihara, K., Tsuzuki, H., & Oka, T. (1968) Arch. Biochem. Biophys. 123, 572.
- Neurath, H., & DeMaria, G. (1950) J. Biol. Chem. 186, 653.
- Nishino, N., & Powers, J. C. (1978) Biochemistry 17, 2846.
- Oney, I., & Caplow, M. (1967) J. Am. Chem. Soc. 89, 6972.
- Petra, P. H. (1970) Methods Enzymol. 19, 460.
- Schechter, I., & Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157.
- Segel, I. H. (1975) *Enzyme Kinetics*, pp 150–160, Wiley, New York.
- Suda, H., Aoyagi, T., Takeuchi, T., & Umezawa, H. (1972) J. Antibiot. 26, 621.
- Umezawa, S., Tatsuta, K., Izawa, O., & Tschiya, T. (1972) Tetrahedron Lett., 97.
- 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.