Glasstone, S., Laidler, K. J., & Eyring, H. (1941) *The Theory of Rate Processes*, McGraw-Hill, New York.
Havel, R. J., & Gordon, R. S. (1960) *J. Clin. Invest. 39*, 1777–1790.
Hegazi, M. F., Quinn, D. M., & Schowen, R. L. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) pp 355–428, Plenum Press, New York.
Iverius, P. H., Lindahl, U., Egelrud, T., & Olivecrona, T. (1972) *J. Biol. Chem. 247*, 6610–6616.
Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp 250–253, McGraw-Hill, New York.
Kannel, W. B., Castelli, W. P., & Gordon, T. (1979) *Ann. Intern. Med. 90*, 85–91.
Kinnunen, P. K. J. (1977) *Med. Biol. 55*, 187–191.
Lecroisey, A., & Kiel, B. (1983) *Biochem. Biophys. Res. Commun. 112*, 907–910.
Matsuoka, N., Shirai, K., & Jackson, R. L. (1980) *Biochim. Biophys. Acta 620*, 308–316.
Miller, G. J. (1980) *Annu. Rev. Med. 31*, 97–108.
Quinn, D. M., Elrod, J. P., Ardis, R., Friesen, P., & Schowen, R. L. (1980) *J. Am. Chem. Soc. 102*, 5358–5365.
Quinn, D. M., Shirai, K., Jackson, R. L., & Harmony, J. A. K. (1982) *Biochemistry 21*, 6872–6879.
Quinn, D. M., Shirai, K., & Jackson, R. L. (1983) *Prog. Lipid Res. 22*, 35–78.
Rawn, J. D., & Lienhard, G. E. (1974) *Biochemistry 13*, 3124–3130.
Rosenberry, T. L. (1975a) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 103–218.
Rosenberry, T. L. (1975b) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3834–3838.
Salomaa, P., Schaleger, L. L., & Long, F. A. (1964) *J. Am. Chem. Soc. 86*, 1–7.
Schowen, K. B. J. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) pp 225–283, Plenum Press, New York.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 109–111, Wiley, New York.
Shirai, K., & Jackson, R. L. (1982) *J. Biol. Chem. 257*, 1253–1258.
Stroud, R. M. (1974) *Sci. Am. 231*, 74–88.
Sugiura, M., & Oikawa, T. (1980) *Chem. Pharm. Bull. 28*, 2803–2806.
Tall, A. R., & Small, D. M. (1980) *Adv. Lipid Res. 17*, 1–51.
Vainio, P., Virtanen, J. A., & Kinnunen, P. K. J. (1982) *Biochim. Biophys. Acta 711*, 386–390.
Vainio, P., Virtanen, J. A., Kinnunen, P. K. J., Gotto, A. M., Sparrow, J. T., Pattus, F., Bougis, P., & Verger, R. (1983a) *J. Biol. Chem. 258*, 5477–5482.
Vainio, P., Virtanen, J. A., Sparrow, J. T., Gotto, A. M., & Kinnunen, P. K. J. (1983b) *Chem. Phys. Lipids 33*, 21–32.
Zilversmit, D. B. (1977) *Adv. Exp. Med. Biol. 109*, 45–59.
Zilversmit, D. B. (1979) *Circulation 60*, 473–485.

Clostridium histolyticum Collagenase: Development of New Thio Ester, Fluorogenic, and Depsipeptide Substrates and New Inhibitors†

Charles F. Vencill, David Rasnick, Katherine V. Crumley, Norikazu Nishino, and James C. Powers*
School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332
Received July 13, 1984; Revised Manuscript Received November 27, 1984

ABSTRACT: A new series of thio ester, depsipeptide, and peptide substrates have been synthesized for the bacterial enzyme *Clostridium histolyticum* collagenase. The hydrolysis of the depsipeptide substrate was followed on a pH stat, and thio ester hydrolysis was measured by inclusion of the chromogenic thiol reagent 4,4′-dithiopyridine in the assay mixture. The best thio ester substrate, Boc-Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba, had a k_{cat}/K_M of 63 000 M^{-1} s^{-1}, while several shorter thio ester sequences were inactive as substrates. In general, the peptide analogues of all the reactive thio ester substrates were shown to be hydrolyzed 5–10 times faster by collagenase. In one case (Z-Gly-Pro-Leu-Gly-Pro-NH_2) where a comparison was made, the peptide substrate was respectively 8- and 106-fold more readily hydrolyzed than the corresponding thio ester and ester substrates. Cleavages of the two fluorescence-quench substrates Abz-Gly-Pro-Leu-Gly-Pro-Nba and Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba could be easily followed fluorogenically since a 5–10-fold increase in fluorescence occurred upon hydrolysis. The fluorescent peptide substrate is the best synthetic substrate known for *C. histolyticum* collagenase with a k_{cat}/K_M value of 490 000 M^{-1} s^{-1}. A series of new reversible inhibitors were developed by the attachment of zinc ligating groups (hydroxamic acid, carboxymethyl, and thiol) to various peptide sequences specific for *C. histolyticum* collagenase. The shorter peptides designed to bind to either the P_3–P_1 or P_1'–P_3' subsites were poor to moderate inhibitors. The thiol $HSCH_2CH_2CO$-Pro-Nba had the lowest K_I (0.02 mM). Elongation of *N*-hydroxy peptide sequences to interact with the P_3–P_3' subsites of the enzyme failed to yield better inhibitors. None of the potential irreversible inhibitor structures, which contained $ClCH_2CO$— or CH_2=CH—CO— groups attached to peptides, proved to be effective.

Collagenases are a small group of highly specific proteases capable of causing hydrolytic cleavage in the triple-helical region of the collagen molecule. In contrast to tissue collagenases, which cleave the collagen helix at a single site, bacterial collagenase will make multiple cleavages. *Clostridium histolyticum* collagenase (EC 3.4.24.3) is one of the most widely studied bacterial enzymes capable of cleaving collagen.

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It requires both zinc and calcium and is inhibited by chelating agents (EDTA[1] and 1,10-phenanthroline), making it a member of the metalloprotease family. Numerous investigators have shown this enzyme capable of cleaving the X–Y bond in the sequence Pro-X-Y-Pro in both native collagen and synthetic peptide substrates (Mandl et al., 1958; Grassman et al., 1961; Yagisawa et al., 1965; Soberano & Schoellman, 1972; Van Wart & Steinbrink, 1981). X can be virtually any amino acid, but Y must be a small nonpolar amino acid (alanine or glycine). Exceptions to this specificity have been reported, but they are probably due to the heterogeneity of most enzyme preparations (Lwebuga-Mukasa et al., 1976) that have been shown to be composed of six forms of the enzyme with molecular weights from 68 000 to 125 000 (Bond & Van Wart, 1984b).

C. histolyticum collagenase assays that use collagen as a substrate are based on changes in physical properties of native collagen such as viscosity (Seifter & Gallop, 1962), optical rotation (Seifter & Gallop, 1962), and opacity (Lapiere & Gross, 1963) or the use of radioactive (^{14}C- or ^{3}H-labeled) collagen to monitor activity (Robertson et al., 1972; Sakamoto et al., 1972). Fluorescent assays have been developed by the attachment of fluorescein to native collagen (Stevens et al., 1975). Most assays using synthetic substrates are stop-point assays and use ninhydrin or peptides containing chromogenic groups (Weinsch & Heidrich, 1963). The most useful and only continuous assay is the spectrophotometric assay developed by Van Wart & Steinbrink (1981).

A few inhibition studies of *C. histolyticum* collagenase have appeared. Several groups tested different peptide fragments as reversible inhibitors of the enzyme (Yaisawa et al., 1965). Most synthetic inhibitors for other metalloproteases have a peptide structure, which is recognized by a particular enzyme, attached to a functional group that can act as a ligand for the active site zinc (Holmquist & Vallee, 1979; Cushman et al., 1977; Nishino & Powers, 1979). This approach has been utilized by Galardy & Grobelny (1983), who found that inhibition of collagenase could be increased by attachment of various phosphoryl derivatives to various peptide fragments.

The purpose of this paper is to further explore the mechanism and specificity of *C. histolyticum* collagenase with new and more sensitive synthetic substrates and to develop new inhibitors. The substrates were designed to incorporate an ester or a thio ester at the scissle bond (X-Gly) of peptide sequences that had previously been shown to be substrates for *C. histolyticum* collagenase. Enzymatic hydrolysis of thio esters is often much faster than that of the corresponding amide and is easily followed by addition of 4,4′-dithiopyridine to the assay mixture (Grassetti & Murray, 1967). Another type of peptide substrate investigated was peptides containing fluorescent groups and fluorescence quenching groups. Cleavage of an internal peptide bond in the substrate results in a large increase in fluorescence as the fluorescent group is separated from the quenching group in solution. Reversible inhibitors containing thiol, carboxymethyl, and hydroxamic acids attached to appropriate peptide sequences were found to inhibit *C. histolyticum* collagenase. A series of potential irreversible inhibitors were designed to search the active site for a reactive nucleophile, but a variety of peptides containing $ClCH_2CO$ and $CH_2{=}CHCO$ groups failed to inhibit *C. histolyticum* collagenase.

[1] Abbreviations: EDTA, ethylenediaminetetraacetate; Z, benzyloxycarbonyl; Abz, 2-aminobenzoyl; Nba, 4-nitrobenzylamide; FA, furoylacryloyl; Boc, *tert*-butyloxycarbonyl; OBzl, benzyl ester; THF, tetrahydrofuran; Me_2SO, dimethyl sulfoxide; DMF, dimethylformamide.

Materials and Methods

Thermolysin, *C. histolyticum* collagenase (type VII), and porcine pancreatic elastase were obtained from the Sigma Chemical Co. The *C. histolyticum* collagenase used was of the highest purity available (1100–1600 units/mg protein), and the concentration was calculated from the number of enzyme units/mL such that 115 units/mL was 1.0 μM (approximately 0.1 mg/mL). A molecular weight of 105 000 was used for the enzyme. Sigma type VII collagenase is reported to contain among the lowest contaminating Bz-Arg-OEt and casein hydrolyzing activities of any commercial collagenase preparation (Bond & Van Wart, 1984a). All amino acids and derivatives were purchased from either Sigma Chemical Co., St. Louis, MO, Chemical Dynamics Corp., South Plainfield, NJ, or Bachem, Torrance, CA. All chemical reagents and buffers were purchased from Aldrich Chemical Co., Milwaukee, WI. Silica gel (32–63 μm) and silica gel 60 were purchased from Sigma Chemical Co.

The purity of all new compounds was checked by thin-layer chromatography (TLC) on Merck silica gel plates in the following systems: R_f^1, chloroform–methanol (3:1 v/v); R_f^2, chloroform–methanol (5:1 v/v); R_f^3, chloroform–methanol (9:1 v/v); R_f^4, chloroform, R_f^5 1-butanol–acetic acid–water (4:1:1 v/v). All NMR spectra were obtained on a Varian T60A instrument and were consistent with the assigned structures. Infrared spectra were measured on a Beckman 4240 instrument. Elemental analyses were performed by Atlantic Microlabs of Atlanta, GA. All melting points were uncorrected. Mercaptans were detected with a nitroprusside reagent. Hydroxamic acids were detected in a ferric chloride solution.

Abbreviated syntheses of the best thio ester substrate, the best fluorogenic substrate, and the best reversible inhibitor are given later in this section. The syntheses of Z-Gly-Gly-Leu-NHOH, Z-Gly-Leu-NHOH, HONHCOCH(i-Bu)CO-Ala-Gly-NH_2, and Z-Gly-Pro-Leu-OH have previously been reported (Nishino & Powers, 1978; Yagisawa et al., 1965). The syntheses of all other new compounds reported in this paper are included in the supplementary material (see paragraph at end of paper regarding supplementary material).

Thio Ester Substrate Assays. The rates of hydrolysis of the thio esters were measured with 4,4′-dithiodipyridine (Grassetti & Murray, 1967). All assays were conducted at 25 °C in 50 mM Tricine, pH 7.5, containing 10 mM $CaCl_2$, 0.48% Me_2SO, and 2.40% MeOH. A typical assay is as follows: to a cuvette containing 2.0 mL of buffer was added 10 μL of a Me_2SO solution of 4,4′-dithiodipyridine, 50 μL of a MeOH solution of the substrate, and 10 μL of collagenase in buffer. The increase in absorbance was monitored with a Beckman Model 35 spectrophotometer. An ϵ of 19 800 M^{-1} cm^{-1} at 324 nm was used (Grassetti & Murray, 1967). Background hydrolysis was negligible in most cases, but when significant, it was corrected for by including both substrate and thiol reagent in the reference cuvette.

The kinetic constants were determined by Hanes plots (Hanes, 1932) and are based on duplicate rate determinations at five or six separate substrate concentrations. All correlation coefficients were greater than 0.99. In the cases where the slope of the Hanes plot was near 0, the slope of a Lineweaver–Burk plot was used to obtain k_{cat}/K_M. This occurred when the substrate solubility was much less than K_M.

pH-Stat Assay of C. histolyticum Collagenase Activity. A typical assay was performed at pH 7.5 with a Radiometer instrument equipped with a nitrogen inlet. The concentration

of titrating NaOH was 0.1 M. To 20 mL of an aqueous solution containing 2.5% methanol (v/v) of Z-Gly-Pro-Leu-OCH_2COPro-NH_2 was added 100 μL of *C. histolyticum* collagenase (5.4 μM in 50 mM Tricine and 10 mM $CaCl_2$, pH 7.5). Nitrogen was continuously bubbled through the reaction mixture to prevent the dissolution of carbon dioxide during the assay. The addition of NaOH to maintain pH 7.5 in the assay solution was followed with time after the addition of enzyme. The kinetic values were determined as previously described for the thio esters.

Ninhydrin Assays of C. histolyticum Collagenase Activity. The hydrolysis reaction was carried out in 2.0 mL of Tricine buffer [52 mM Tricine, 10.5 mM $CaCl_2$, 0.5% Me_2SO (v/v), pH 7.5] to which was added 50 μL of substrate in methanol. The reaction was initiated by the addition of 10 μL of *C. histolyticum* collagenase (0.5 μM) in buffer. At appropriate time intervals, 0.25-mL aliquots were removed to test tubes containing an equal volume of 0.1 M HCl to stop the reaction. The amount of α-amino group released over time was determined colorimetrically according to the ninhydrin method of Rosen (1957). The kinetic values were determined as previously described for the thio esters. Glycylprolinamide or glycylproline 4-nitrobenzylamide was used as the ninhydrin color standards; hydrolysis product concentrations were determined from curves of absorbance at 570 nm vs. standard concentration. Fresh Tricine buffer was prepared after four to five assays since the background A_{570} increased as the buffer aged.

Fluorescent Assays. The assay procedure for both Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba and Abz-Gly-Pro-Leu-Gly-Pro-Nba was the same. All assays were conducted at 25 °C in 50 mM Tricine, pH 7.5, containing 10 mM $CaCl_2$, 0.48% Me_2SO (v/v), and 2.40% MeOH (v/v). A typical assay is as follows: to a cuvette containing 2 mL of buffer was added 10 μL of Me_2SO, 50 μL of a MeOH solution of the substrate, and 10 μL of collagenase in buffer. The fluorescence change was measured continuously on a Perkin-Elmer fluorescence spectrophotometer and Model 50 recorder. The excitation and emission wavelengths were 340 and 415 nm, respectively. The hydrolysis rates were linear up to 15%.

Fluorescence intensity vs. concentration of substrates (and hydrolysis products) curves were constructed for each peptide tested (see Figure 1 for an example). The fluorescent change at each substrate concentration was measured by subtracting the tangent of the starting peptide curve from the tangent of the hydrolysis product curve at the appropriate concentration of substrate and hydrolysis products. These tangents usually fell in the linear portion of the starting peptide curve and always in the linear region of the hydrolysis products' curve. The fluorescence change was used to convert fluorescence rate measurements into absolute molar velocities. Quinine sulfate (1 μM in 0.10 N HCl) was assigned the value of 1 fluorescence unit and was used to standardize the instrument daily. The kinetic constants were determined as previously described for the thio esters.

Assay of the Substrate FA-Leu-Gly-Pro-Ala. Kinetic measurements with this substrate were as previously described (Van Wart & Steinbrink, 1981). In a typical experiment 10 μL of collagenase was added to 2.0 mL of a pH 7.5 solution of FA-Leu-Gly-Pro-Ala containing 50 mM Tricine, 10.1 mM $CaCl_2$, 0.48% Me_2SO and 2.4% methanol (v/v). The decrease in absorbance over time was monitored with a Beckman Model 35 spectrophotometer. The wavelengths used at different substrate concentrations were 345 (0.96 mM), 343 (0.63–0.34 mM), and 338 nm (0.24–0.19 mM). The kinetic values were determined by the Hanes method (Hanes, 1932); the correlation coefficient was 0.995.

Reversible Inhibitor Assays. The reversible inhibitors were assayed by one of two procedures. The first involved use of the fluorogenic peptide Abz-Gly-Pro-Leu-Gly-Pro-Nba. The procedure is as follows: to 2.0 mL of a substrate (Abz-Gly-Pro-Leu-Gly-Pro-Nba) solution [pH 7.5, 50 mM Tricine, 10 mM $CaCl_2$, 0.5% Me_2SO (v/v)] of the appropriate concentration (0.15–0.025 mM) was added 50 μL of a MeOH solution of the inhibitor and 10 μL of collagenase in buffer. The fluorescence change was monitored continuously on a Perkin-Elmer fluorescence spectrophotometer and a Model 50 recorder. The second assay involved the use of the depsipeptide Z-Gly-Pro-Leu-OCH_2CO-Pro-NH_2. To 1 mL of substrate (Z-Gly-Pro-Leu-OCH_2CO-Pro-NH_2) solution of the appropriate concentration (1 or 4 mM) was added 100 μL of the appropriate inhibitor solution (1–10 mM). The pH was adjusted to 7.5, and 50 μL of collagenase solution (10 mM in calcium acetate) was added to give a final enzyme concentration of 60 μg/mL.

The kinetic constants were determined by both Lineweaver–Burk and Dixon plots. They are based on duplicate rate determinations at five or six separate substrate and inhibitor concentrations. All correlation coefficients were greater than 0.99. Most of the K_I values for the hydroxamic acids were determined with the depsipeptide Z-Gly-Pro-Leu-OCH_2CO-Pro-NH_2. The inhibition constants of $HSCH_2CH_2CO$-Pro-Nba and $HO_2CCH_2NHCH_2CO$-Pro-Nba were determined with FA-Leu-Gly-Pro-Ala as a substrate. All other mercaptan inhibitor constants were determined from the fluorescent assay with Abz-Gly-Pro-Leu-Gly-Pro-Nba.

Irreversible Inhibitor Assays. The procedure for treating *C. histolyticum* collagenase with all the potential alkylating agents was the same. To a 1.1-mL solution of the enzyme (50 mM Tricine, 10 mM $CaCl_2$, pH 7.5) was added 25 μL of a methanol solution of the alkylating agent. Incubation with all the alkylating agents was continued for no less than 2 h. An incubation mixture containing the enzyme but no alkylating agent was shown to be stable for 12 h. Aliquots (100 μL) were periodically taken and assayed for residual enzyme activity by following the hydrolysis of a 2.16-mL solution of Z-Gly-Pro-Leu-SCH_2CO-Pro-Nba [0.045 mM, 0.27 mM 4,4′-dithiodipyridine, 50 mM Tricine, 10 mM $CaCl_2$, 0.46% Me_2SO (v/v), 2.4% MeOH (v/v)] spectrophotometrically at 324 nm on a Beckman Model 35 spectrophotometer.

3-Mercaptopropionyl-L-proline 4-Nitrobenzylamide ($HSCH_2CH_2CO$*-Pro-Nba).* Ac-$SCH_2CH_2CO_2H$ was synthesized from acetic anhydride and 3-mercaptopropronic acid. Pro-Nba·HCl was synthesized by standard peptide condensation methods. The two fragments (3-acetylmercapto)-propionic acid (1.03 g, 7 mmol) and Pro-Nba·HCl (2.00 g, 7 mmol) were then coupled by the mixed-anhydride method with isobutyl chloroformate and *N*-methylmorpholine in THF–DMF (1:1 v/v). The crude product was recrystallized from cold ether: yield 1.72 g (64%); R_f^2 0.67; mp 91–92 °C.

The recrystallized Ac-SCH_2CH_2CO-Pro-Nba (1.13 g, 3 mmol) was dissolved in 25 mL of anhydrous methanol and cooled to 0 °C. Ammonia gas was bubbled through this solution for 5 min. After 10 min, the reaction was complete. The reaction mixture was evaporated and redissolved in CH_2Cl_2. This solution was washed 3 times with 10% citric acid, 3 times with 5% $NaHCO_3$, and 3 times with a saturated NH_4Cl solution. Purification of the crude product was achieved via flash column chromatography on silica gel (32–63 μm) with CH_2Cl_2–MeOH (20:1 v/v) as the eluant: yield 0.657

Table I: Kinetic Constants for Hydrolysis of Synthetic Substrates for *C. histolyticum* Collagenase

substrates	assay[a]	[S] range (μM)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Ac-SCH_2CO-Pro-Nba	thio, fluor	200			NH
Ac-SCH_2CH_2CO-Pro-Nba	thio	200			NH
Z-Gly-Pro-Leu-SCH_3	thio, nin	230			NH
Ac-Leu-SCH_2CO-Pro-Nba	thio	100			NH
Z-Gly-Pro-Leu- Gly -Pro-NH_2	nin	180–540			35 000
Z-Gly-Pro-Leu-SCH_2CO-Pro-NH_2	thio	39–260			4 200
Z-Gly-Pro-Leu-OCH_2CO-Pro-NH_2	pH stat	510–1500	1.6	0.54	330
Z-Gly-Pro-Leu- Gly -Pro-Nba	nin	80–240			140 000
Z-Gly-Pro-Leu-SCH_2CO-Pro-Nba	thio	25–100			16 000
Boc-Abz-Gly-Pro-Leu- Gly -Pro-Nba	nin	25–92			340 000
Boc-Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba	thio	5–27			63 000
Abz-Gly-Pro-Leu- Gly -Pro-Nba	nin	47–140	0.24	82	340 000
	fluor	40–90	0.14	68	490 000
Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba	thio	40–240	0.26	8.1	32 000
	fluor	34–246			42 000
Abz-Pro-Leu -Gly -Pro-Nba	fluor				NH
FA-Leu -Gly -Pro-Ala	spec	190–960	0.45	22	48 000

[a] Abbreviations: thio, determined by thioester assay; fluor, determined by the fluorometric assay; nin, determined by ninhydrin assay; pH stat, determined by pH stat assay; spec, determined by spectrophotometric assay of Van Wart & Steinbrink (1981), who report K_M = 0.5 mM and k_{cat} = 19 s^{-1} under slightly different conditions. Complete experimental details are under Materials and Methods. [b] NH, no hydrolysis was observed.

g (65%); R_f^2 0.59; mp 110–111 °C. Anal. Calcd for $C_{15}H_{19}N_3O_4S$: C, 53.39; H, 5.69; N, 12.46. Found: C, 53.36; H, 5.72; N, 12.41.

*2-Aminobenzoylglycyl-*L*-prolyl-*L*-leucyl-2-mercaptoacetyl-*L*-proline 4-Nitrobenzylamide (Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba)*. Both Boc-Abz-Gly-Pro-OH and Leu-SCH_2CO-Pro-Nba·HCl were synthesized by standard peptide condensation methods. These two fragments Boc-Abz-Gly-Pro-OH (0.782 g, 2 mmol) and Leu-SCH_2CO-Pro-Nba·HCl (0.944 g, 2 mmol) were then coupled by the mixed anhydride method. Purification of the crude product was achieved via flash column chromatography on silica gel (32–63 μm) with MeOH–ether (1:20 v/v) as the eluant: yield 0.612 g (37%); R_f^2 0.79; mp 120–130 °C dec. Anal. Calcd for $C_{39}H_{51}N_7O_{10}S·H_2O$: C, 56.56; H, 6.46; N, 11.84; S, 3.87. Found: C, 56.68; H, 6.47; N, 11.82; S, 3.84.

The purified Boc-Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba (0.41 g, 0.5 mmol) was then dissolved in dry CH_2Cl_2 (2.31 mL). To this solution was added 15 equiv of trifluoroacetic acid (0.57 mL, 7.5 mmol). The reaction was complete after 45 min. The mixture was concentrated, dissolved in THF, and added to a dioxane solution containing 2.0 equiv of HCl (1.0 mL, 1 N HCl/dioxane). The product was filtered and dried in a vacuum desiccator with P_2O_5 and KOH. Further purification of the crude product was achieved via flash column chromatography on silica gel (32–63 μm) with CH_2Cl_2–MeOH–ether (12:2:3 v/v/v) as the eluant: yield 0.203 g (56%); R_f^2 0.72; mp 100–110 °C dec. Anal. Calcd for $C_{34}H_{43}N_7O_8S·^1/_2HCl$: C, 56.08; H, 6.03; N, 13.47; S, 4.40. Found: C, 55.89; H, 6.08; N, 13.42; S, 4.39.

*2-Aminobenzoylglycyl-*L*-prolyl-*L*-leucylglycyl-*L*-proline 4-Nitrobenzylamide (Abz-Gly-Pro-Leu-Gly-Pro-Nba)*. Boc-Abz-OH was synthesized from 2-[((*tert*-butyloxycarbonyl)-oxy)imino]-2-phenylacetonitrile (Boc-ON) and 2-aminobenzoic acid. Gly-Pro-Leu-Gly-Pro-Nba·HCl was synthesized by standard peptide condensation methods. The two fragments Boc-Abz-OH (0.47 g, 2 mmol) and Gly-Pro-Leu-Gly-Pro-Nba·HCl (0.912 g, 2 mmol) were coupled by using triethylamine (0.21 mL, 1.5 mmol), *N*-hydroxybenzotriazole (0.200 g, 1.5 mmol), and *N,N'*-dicyclohexylcarbodiimide (0.340 g, 1.65 mmol) in 6 mL of DMF. After a standard workup, the crude product was further purified via column chromatography on silica gel 60 with MeOH–$CHCl_3$ (1:24 v/v) as the eluant. The product was recrystallized from MeOH–ether: yield 0.875 g (74%); R_f^2 0.63. Anal. Calcd for $C_{39}H_{52}N_8O_{10}·^3/_2H_2O$: C, 57.06; H, 6.75; N, 13.65. Found: C, 57.29; H, 6.61, N, 13.45.

The purified Boc-Abz-Gly-Pro-Leu-Gly-Pro-Nba (0.400 g, 0.5 mmol) was dissolved in 4 mL of dioxane. To this solution was added 1 mL of 5 N HCl/dioxane at 25 °C. After 1 h the reaction was complete. The reaction mixture was concentrated and dried in a vacuum desiccator with P_2O_5 and KOH. Purification of the crude product was achieved via column chromatography on silica gel 60 with MeOH–$CHCl_3$ (1:24 v/v) as the eluant. The product was recrystallized from CH_2Cl_2–ether: yield 0.20 g (58%); R_f^2 0.58; mp 120–121 °C. Anal. Calcd for $C_{34}H_{45}N_8O_8Cl$: C, 55.99; H, 6.23; N, 15.37. Found: C, 56.04; H, 6.26; N, 15.36.

Results

One of our first goals in the study of *C. histolyticum* collagenase was the development of a sensitive and convenient assay for this enzyme. Prior to our work, the most useful rate assay was the chromophoric assay developed by Van Wart & Steinbrink (1981) using furoylacryloyl-L-leucylglycyl-L-prolyl-L-alanine (FA-Leu-Gly-Pro-Ala). This substrate was synthesized and assayed at pH 7.5 and under the same conditions as the new substrates reported here. The kinetic parameters obtained for FA-Leu-Gly-Pro-Ala are included in Table I for comparison with our substrates. Initially, we investigated an ester substrate for collagenase.

Esterase Activity of C. histolyticum Collagenase. The depsipeptide Z-Gly-Pro-Leu-OCH_2CO-Pro-NH_2, an analogue of the excellent collagenase substrate Z-Gly-Pro-Leu-Gly-Pro-OH (Yagisawa et al., 1965), was synthesized by standard methods. Cleavage of the depsipeptide (1 mM) by *C. histolyticum* collagenase (53 μM) at pH 7.2 (10 mM Tris, 10 mM

$CaCl_2$, 1% MeOH) and 25 °C was investigated by TLC. During the time course of the incubation, the spot for the depsipeptide (R_f[1] 0.65) diminished while a new spot (R_f[1] 0.26) identical with glycolylprolinamide appeared and increased in intensity. No change was observed if the incubation mixture lacked enzyme, if the enzyme solution was boiled for 1 min prior to addition to the reaction mixture, or if 10 mM 1,10-phenanthroline was present in the incubation mixture. In a pH 8.8 Tris buffer, cleavage of the depsipeptide was observed in both the presence and the absence of enzyme.

The hydrolysis of Z-Gly-Pro-Leu-OCH_2CO-Pro-NH_2 could be conveniently followed by using a pH stat at pH 7.5 in a solution containing either 2% DMF or 2% MeOH to solubilize the substrate. The K_M for the substrate was determined to be 1.6 mM at pH 7.5. The hydrolysis of the ester (10.2 mM, 2% DMF) is linearly dependent on enzyme concentration over the range studied. The assay is easily usable down to 10 μg/mL (17 μM) enzyme in the assay solution, with a convenient concentration being 50 μg/mL (87 μM).

The pH dependence of K_M for the collagenase-catalyzed hydrolysis of Z-Gly-Pro-Leu-OCH_2CO-Pro-NH_2 was measured and K_M decreased as the pH increased. A negligible decrease in hydrolysis rate of peptides due to denaturation of the enzyme has been observed in the pH range (6.0–8.5) studied (Yagisawa et al., 1965). Since the substrate is neutral, the decrease in K_M value indicates that a dissociable group with a pK_a of ca. pH 7 is affecting the binding of the ester to the enzyme. A similar pH dependence of the hydrolysis of Z-Gly-Pro-Leu-Gly-Pro-OH by collagenase has been observed by Yagisawa et al. (1965).

To eliminate the possibility that the hydrolysis of the depsipeptide was due to contaminating serine proteases in the collagenase, the enzyme was pretreated with 10 mM phenylmethanesulfonyl fluoride for 10 min at pH 7.6. Under a depsipeptide concentration of 10.2 mM in 2% DMF and an enzyme concentration of 71 μg/mL (0.123 mM), no change in hydrolysis rate was observed at pH 7.5. No ester hydrolysis was observed if the assay mixture contained the metalloprotease inhibitor 1,10-phenanthroline (8.6 mM in 4% DMF to dissolve the inhibitor). The depsipeptide was rapidly hydrolyzed by porcine pancreatic elastase (K_M = 6.4 mM, k_{cat} = 0.18 s^{-1}). Thermolysin does not measurably cleave the ester bond of Z-Gly-Pro-Leu-OCH_2CO-Pro-NH_2 as determined with the pH stat. However, TLC analysis indicated that the substrate was cleaved rapidly by thermolysin, presumably at the Pro–Leu bond.

Thioesterase and Peptidase Activity of C. histolyticum Collagenase. Thio ester hydrolysis rates were measured continuously by reaction of the released thiol with the 4,4′-dithiodipyridine present in the reaction mixture. The product of this reaction, 4-thiopyridone, has an extremely high extinction coefficient (19 800 M^{-1} cm^{-1} at 324 nm) (Grassetti & Murray, 1967). The effect of the 4,4′-dithiodipyridine concentration on the hydrolysis rates of the thio esters was tested (see Table III of supplementary material). The concentration of 4,4′-dithiodipyridine was varied between 2 and 10 times the concentration of the substrate (Z-Gly-Pro-Leu-SCH_2CO-Pro-Nba), and no effect on the rate of hydrolysis was observed. The effect of organic solvent was also determined (see Table IV of supplementary material). Both DMF and Me_2SO have a considerable effect (41% and 49% decrease, respectively) on the rate of hydrolysis at a concentration of 2%. Methanol has almost no effect up to concentrations of 3% (v/v). The concentrations of these solvents selected for the thio ester substrate assays (2.40% MeOH, 0.48% Me_2SO) were chosen to maximize the solubility of the substrates and minimize the deleterious effects these solvents have on the hydrolysis rates. The effect of various buffers was also tested for collagenase. At a concentration of 98 mM, the relative rates were as follows: HEPES, 39; Tris-HCl, 67; Tricine, 100. As was observed by Van Wart (Van Wart & Steinbrink, 1981), Tricine is the best buffer for collagenase.

The kinetic constants for the hydrolysis of the thio ester and peptide substrates are given in Table I. As is generally found with collagenase, the shorter thio ester substrates (Ac-SCH_2CO-Pro-Nba, Ac-SCH_2CH_2CO-Pro-Nba, Z-Gly-Pro-Leu-SCH_3, and Ac-Leu-SCH_2CO-Pro-Nba) were not hydrolyzed by the enzyme. The dipeptide Z-Gly-Pro-OH was added to the assay containing Ac-SCH_2CO-Pro-Nba in an attempt to see if binding to the P_3[2] and P_2 subsites on the enzyme concomitant with binding of the substrate at P_1', P_2', and P_3' would initiate the hydrolysis of the thio ester. Addition of Pro-NH_2 to the assay containing Z-Gly-Pro-Leu-SCH_3 was again an attempt to initiate hydrolysis of the thio ester by binding to the P_2' subsite. In neither case was hydrolysis of the thio ester observed.

All thio esters of the form X-Gly-Pro-Leu-SCH_2CO-Pro-Y were hydrolyzed by the enzyme. However, the peptide analogues of the thio esters were 5–10-fold more reactive in each case. The best thio ester substrate sequences Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba (k_{cat}/K_M = 32 000 M^{-1} s^{-1}) and Boc-Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba (k_{cat}/K_M = 63 000 M^{-1} s^{-1}) proved to be the best peptide substrate sequences Abz-Gly-Pro-Leu-Gly-Pro-Nba (k_{cat}/K_M = 340 000 M^{-1} s^{-1}) and Boc-Abz-Gly-Pro-Leu-Gly-Pro-Nba (k_{cat}/K_M = 340 000 M^{-1} s^{-1}) while the poorest thio ester and depsipeptide sequences Z-Gly-Pro-Leu-SCH_2CO-Pro-NH_2 (k_{cat}/K_M = 4200 M^{-1} s^{-1}) and Z-Gly-Pro-Leu-OCH_2CO-Pro-NH_2 (k_{cat}/K_M = 320 M^{-1} s^{-1}) proved to be the poorest peptide substrate sequence Z-Gly-Pro-Leu-Gly-Pro-NH_2 (k_{cat}/K_M = 35 000 M^{-1} s^{-1}). The fourth peptide substrate tested (Z-Gly-Pro-Leu-Gly-Pro-Nba, k_{cat}/K_M = 140 000 M^{-1} s^{-1}) fell as expected in the range between these two extremes.

Fluorescent Substrate Assays. A method previously utilized for fluorescent assays for carboxypeptidase (Latt et al., 1972), trypsin (Carmel et al., 1973), angiotensin converting enyzme (Persson & Wilson, 1977), enzymes associated with blood clotting (Castillo et al., 1983), and *Pseudomonas aeruginosa* elastase (Nishino & Powers, 1980) was applied to collagenase. A fluorescent group (2-aminobenzoyl, Abz) and a quenching group (4-nitrobenzylamide, Nba) were attached to a previously tested substrate for collagenase (Z-Gly-Pro-Leu-Gly-Pro-OH; Yagisawa et al., 1965). In the intact peptide the fluorescent group is in close proximity to the quenching group. Hydrolysis of the peptides separates the fluorescent group from the quenching group resulting in an increase in the fluorescence. Three peptides based on this principle have been synthesized for *C. histolyticum* collagenase. Both Abz-Gly-Pro-Leu-Gly-Pro-Nba and Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba displayed a large increase in fluorescence upon hydrolysis by collagenase. Abz-Pro-Leu-Gly-Pro-Nba proved not to be a substrate for collagenase. The intact peptides have a small amount of intrinsic fluorescence (Figure 1) probably caused by incomplete quenching due to the conformation of the peptide or to the nature of the quenching group (Nba). Complete hydrolysis of the peptides led to a 7–10-fold increase

[2] The nomenclature for the individual amino acid residues (P_3, P_2, P_1) of a substrate and for the subsites (S_3, S_2, and S_1) of an enzyme is that of Schechter & Berger (1967).

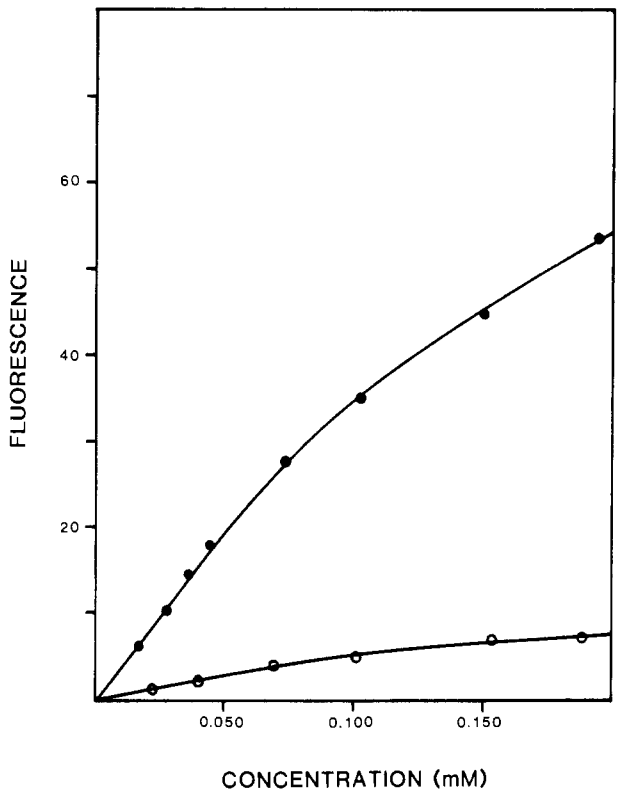

FIGURE 1: Fluorescence of Abz-Gly-Pro-Leu-Pro-Nba (open circles) and its hydrolysis products (Abz-Gly-Pro-Leu-OH and H-Gly-Pro-Nba) after total digestion with *C. histolyticum* collagenase (closed circles). Quinine sulfate was used as the fluorescence standard. One fluorescence unit is equal to the fluorescence of a 1.0 μM solution of quinine sulfate in 0.1 N H_2SO_4.

in fluorescence. The degree to which the fluorescence increased was related to the substrate concentration. The kinetic constants for Abz-Gly-Pro-Leu-Gly-Pro-Nba and Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba are given in Table I. Kinetic constants for the first substrate were also determined by a ninhydrin assay and for the second, kinetic constants were also measured using the thio ester assay. In both cases good correspondence between the fluorescence assay and the alternate assays was observed.

Irreversible Inhibitors for C. histolyticum Collagenase. Incubation of *C. histolyticum* collagenase with chloromethyl ketone serine protease inhibitor Ac-Ala-Ala-Pro-IleCH_2Cl (Powers et al., 1977) or the thermolysin inhibitor $ClCH_2CO$-DL-(*N*-OH)Leu-OCH_3 (Rasnick & Powers, 1978) resulted in no loss of activity after 72 h. This is in contrast to the results of Balaevskaya et al. (1981), who reported inhibition by Z-Gly-Pro-LeuCH_2Cl. With the failure of these two inhibitors, the synthesis of a new set of inhibitors that might be specific for collagenase was undertaken. The inhibitor design was centered around collagenase's requirement for a proline residue at P_2'. Various groups known to have an affinity for P_3' (NHBzl and Nba) and AlaOCH_3 (Van Wart & Steinbrink, 1981; Galardy & Grobelny, 1983) were attached to proline to increase its binding affinity. The P_1 and P_1' positions of the active site were then scanned for a nucleophile. Attachment of the four reactive groups $ClCH_2CO$–, CH_2=CHCO—, $ClCH_2CO$-Gly–, and CH_2=CHCO-Gly– to various proline-containing peptides placed an electrophilic center in various parts of the active site of collagenase. This approach failed with *C. histolyticum* collagenase since no noticeable inhibition of the enzyme was observed after incubation for 2 h with any of the inhibitors tested. The inhibitors tested (Table V of supplementary material) were $ClCH_2CO$-Pro-NH_2, $ClCH_2CO$-Pro-Nba, $ClCH_2CO$-Gly-Pro-Nba, $ClCH_2CO$-Gly-Pro-Ala-OMe, $ClCH_2CO$-Gly-Pro-NHBzl, CH_2=CHCO-Pro-Nba, and CH_2=CHCO-Gly-Pro-Nba.

Table II: Kinetics of Reversible Inhibitors of *C. histolyticum* Collagenase

inhibitor	K_I (mM)
$HOCH_2CO$-Pro-NH_2	15
Z-Gly-Pro-Leu-OH	4.3[b]
Z-Gly-Leu-NHOH	1.5
Z-Gly-Gly-Leu-NHOH	18.9
Z-Gly-Pro-Leu-NHOH	0.24
$HONHCOCH_2CO$-Pro-NH_2	4.6
$HONHCOCH_2CH_2CO$-Pro-NH_2	0.44
HONHCOCH(i-Bu)CO-Ala-Gly-NH_2	4.1
Boc-Gly -Pro-Leu-(N-OH)Gly-OEt	none[c]
H-Gly -Pro-Leu-(N-OH)Gly-Pro-OH	none[d]
Boc-Gly -Pro-Leu-(N-OH)Gly-Pro-OH	17
Boc-Gly-Pro-OBzl	0.8
Boc-Gly-Pro-NHBzl	2.2
$HO_2CCH_2NHCH_2CO$-Pro-Nba	1.2
$HSCH_2CH_2CO$-Pro-NH_2	0.25
$HSCH_2CH_2CO$-Pro-NHBzl	0.17
$HSCH_2CH_2CO$-Pro-Nba	0.02

[a] Conditions: pH 7.5, 10 mM Ca^{2+}, 50 mM Tricine, 2.5% MeOH, and 0.5% Me_2SO with the mercaptan inhibitors. No buffer or organic solvents were used for the remaining inhibitors. Collagenase concentration was 10–20 nM. [b] Yagisawa et al. (1965) reported a K_I of 4.9 mM. [c] No inhibition at a maximum concentration of 1 mM. [d] No inhibition at a maximum concentration of 3 mM.

Competitive Inhibitors. The inhibition results obtained with a number of competitive inhibitors are shown in Table II. Both hydrolysis products of the depsipeptide were reasonably good inhibitors of *C. histolyticum* collagenase, with the N-terminal fragment Z-Gly-Pro-Leu-OH having a lower K_I value than the glycolylprolinamide. The best hydroxamic acid inhibitor was Z-Gly-Pro-Leu-NHOH with a K_I of 0.2 mM. This inhibitor contains the P_3–P_1 residues used in a number of the thio ester, depsipeptide, and peptide substrates. As shown by TLC analysis, this peptide was not enzymatically cleaved. Due to a preference of collagenase for a P_2 prolyl residue, Z-Gly-Gly-Leu-NHOH was a poorer inhibitor. The peptide hydroxamic acids corresponding to the P_1'–P_2' residues of good substrates ($HONHCOCH_2CO$-Pro-NH_2 and $HONHCOCH_2CH_2CO$-Pro-NH_2) were also moderately good reversible inhibitors. Placement of the second methylene group between the hydroxamic acid and the prolyl residue decreased the K_I value by a factor of 10. Surprisingly, elongation of the peptide recognition sequence did not produce better K_I values. The three *N*-hydroxy peptides with sequences corresponding to P_3–P_2' and P_3–P_1' residues of good *C. histolyticum* substrates showed no or weak inhibition of this enzyme. Three thiol-containing peptides were synthesized and shown to bind more tightly than the hydroxamic acids. Elongation of the mercaptan peptide by addition of aromatic residues at the P_3' subsite again caused a decrease in the K_I values ($HSCH_2CH_2CO$-Pro-NHBzl, K_I = 0.14 mM; $HSCH_2CH_2CO$-Pro-Nba, K_I = 0.02 mM). This is in line with the increase in k_{cat}/K_M values observed with the thio ester substrates when an aromatic residue was able to bind to the P_3' subsite. In fact, two short peptide inhibitors containing aromatic residues at P_3' (Boc-Gly-Pro-OBzl and Boc-Gly-Pro-NHBzl) were moderate inhibitors. Placement of another

chelating group (carboxyl) into the peptide sequence produced a slightly poorer inhibitor.

Discussion

Peptide thio esters have been shown to be highly sensitive substrates for serine proteases. Over 30 different serine proteases are known to react with thio ester substrates, and thus far, no serine protease has failed to react with one of these substrates [see, for example, Harper et al. (1984) and McRae et al. (1981)].

Serine proteases are mechanistically different from metalloproteases, but since both are involved in the hydrolysis of peptide bonds, it seemed plausible to extend the use of thio esters to metalloendoproteases. Two metalloexopeptidases, leucine aminopeptidase and carboxypeptidase A, have previously been shown to possess thioesterase activity (Metrone, 1972; Suh & Kaiser, 1975). The first thio ester substrate for collagenase (Z-Gly-Pro-Leu-SCH_2CO-Pro-NH_2) synthesized in our lab proved to be an active substrate.

As has been previously observed, peptides must have proline residues located at both the P_2 and P_2' positions to be effective substrates for collagenase (Seifter & Harper, 1971). All of the shorter thio ester substrates synthesized (Ac-SCH_2CO-Pro-Nba, Ac-SCH_2CH_2CO-Pro-Nba, Z-Gly-Pro-Leu-SCH_3, and Ac-Leu-SCH_2CO-Pro-Nba) that lacked this feature displayed no hydrolysis at concentrations of up to 200 μM. In addition, no hydrolysis of these short peptides could be observed when a second proline-containing peptide was added such that the two combined peptides mimicked a normal collagenase substrate containing two prolines. However, all of the substrates of the form X-Gly-Pro-Leu-SCH_2CO-Pro-Y were readily hydrolyzed by collagenase. Replacement of an amide by a *p*-nitrobenzylamide at subsite P_3' increased the hydrolysis rate by a factor of 4. Replacement of a P_4 benzyloxycarbonyl (Z) group with a 2-(*tert*-butyloxycarbonylamino)benzoyl group increased the hydrolysis rate by a factor of 4. Cleavage of the *tert*-butyloxycarbonyl group from the 2-aminobenzoyl group to produce the free amine increased the solubility of the substrate but decreased the hydrolysis rate by a factor of 0.50. Certain aromatic residues (Abz or Nba) seem to have an affinity for both S_4 and S_3' subsites on collagenase.

The best thio ester substrate (Boc-Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba) compares favorably with previously reported chromogenic assays. Both the Z-Gly-Pro-Gly-Gly-Pro-Ala-OH (Grassman & Nordwig, 1960) and Z-Gly-Pro-Leu-Gly-Pro-OH (Nagai et al., 1960) assays follow the production of the free amine by reaction with ninhydrin. Phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg-OH (Wunsch & Heidrich, 1963) hydrolysis is monitored by the extraction of the chromogenic peptide phenylazobenzyloxycarbonyl-Pro-Leu-OH into organic solvents and determination of its concentration colorimetrically. All these assays are time consuming and do not provide a continuous profile of substrate hydrolysis. Until now, FA-Leu-Gly-Pro-Ala was the most sensitive chromogenic peptide (Van Wart & Steinbrink, 1981). Thio ester substrates have three distinct advantages over furoylacryolyl substrates. First, the k_{cat}/K_M value for Boc-Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba is larger, making it one of the more sensitive assays substrates known. Second, the thio ester assay is a continuous assay (like the furoylacryloyl peptide), but the extinction coefficient of the chomophore produced $\epsilon_{324nm} = 19\,800\ M^{-1}\ cm^{-1}$ is 8 times larger than the $\epsilon_{324nm} = 2510\ M^{-1}\ cm^{-1}$ for FA-Leu-Gly-Pro-Ala. Together these two effects make the thio ester Boc-Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba approximately a 10-fold more sensitive substrate than the furoylacryoyl peptide. The third advantage is cleavage specificity; only cleavage at the thio ester bond will produce a chromogenic product so random cleaves of the substrate by contaminating proteases should not affect the assay.

Fluorescent Assays. Prior to this work the only fluorescent assay for *C. histolyticum* collagenase was the assay involving the attachment of fluorescein to native collagen (Stevens et al., 1975). The success achieved previously (Castillo et al., 1983; Nishino & Powers, 1980) with synthetic fluorescent quench peptide substrates stimulated us to use this technique to develop sensitive fluorescence peptidase and thioesterase assays for collagenase. Both Abz-Gly-Pro-Leu-Gly-Pro-Nba and Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba exhibit a large increase in fluorescence upon cleavage of the scissle bond. The peptide Abz-Gly-Pro-Leu-Gly-Pro-Nba ($k_{cat}/K_M = 490\,000\ M^{-1}\ s^{-1}$) is the fastest known substrate for collagenase. The thio ester analogue, although still a good substrate, is 10 times slower. However, when the assay mixture may contain contaminating fluorophores or quenchers or when inhibitors are being tested whose fluorescent properties are unknown, the chromogenic thio ester assay may be preferred. It also should be noted that the hydrolysis of the thio ester Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba can be determined either chromogenically or fluorogenically as the situation requires.

Comparison of the Peptidase, Thioesterase, and Esterase Activity of Collagenase. The peptidase activity of collagenase was in every case 5–100-fold higher than the thioesterase or esterase activity of the thio ester or ester with the corresponding sequence. The thio ester bond is much more reactive than a peptide bond, and we expected to observe the reverse. Indeed, with serine proteases, thio ester substrates are in general the most reactive substrates that have yet been described (Harper et al., 1984). Examination of the kinetic constants for Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba ($K_M = 0.26$ mM, $k_{cat} = 8.1\ s^{-1}$) and Abz-Gly-Pro-Leu-Gly-Pro-Nba ($K_M = 0.24$ mM, $k_{cat} = 82\ s^{-1}$) shows the difference in reactivity to be clearly due to a 10-fold difference in k_{cat}. Similarly, the kinetic results presented here show that for the three substrates of the general form Z-Gly-Pro-Leu-X-Pro-NH_2, where X is Gly, $-SCH_2CO-$, or $-OCH_2CO-$, the order of reactivity is Gly > $-SCH_2CO-$ > $-OCH_2CO-$, where the k_{cat}/K_m value is reduced ca. 10-fold at each step.

The thioesterase activity of two other metalloproteases has been reported. Leucine aminopeptidase has been shown to be capable of hydrolyzing leucine thioethyl ester at a rate 50 times greater than the rate for leucine 4-nitroanilide (Metrione, 1972). The K_M value for the thio ester (1.3 mM) is quite close to that of leucine benzyl ester (1.6 mM) and leucine amide (5.7 mM). Carboxypeptidase A hydrolyzes cinnamoyl α-mercapto-β-phenylpropionate (Suh & Kaiser, 1975) but has been more extensively studied with ester substrates (Vallee et al., 1983). The catalytic efficiency of the enzyme toward esters is not much greater than that toward peptides, the k_{cat}/K_M values for esters being generally much less than 100 times that for peptides and in some cases the same. The overwhelming evidence that esters and peptides are hydrolyzed by different mechanisms has been interpreted to indicate different binding modes for the two types of substrates (Vallee et al., 1983) or differences in rate-limiting steps in the catalytic mechanism. In the case of thermolysin and metalloendoproteases, ester and peptide hydrolysis proceed by essentially identical binding and catalytic mechanisms, with the k_{cat}/K_M values being 3–8-fold slower with esters (Holmquist & Vallee, 1979).

In contrast to both leucine aminopeptidase and carboxypeptidase, we find that collagenase along with thermolysin cleaves peptide substrates substantially faster than esters or thio esters. It is possible that this is due to a slightly different binding mode for ester and amide substrates. Collagenase seems to have a distinct preference for two prolines at P_2 and P_2', and substrates lacking this structural feature in general are poorly hydrolyzed. Replacement of a thio ester bond for an amide in the center of a peptide may prevent correct binding of the substrate with these important subsites of the enzyme. There are substantial bond angle differences between peptide bonds and thio ester or ester bonds (Zacharias et al., 1983). The C(carbonyl)–X bond is shorter (1.32 Å) in peptides than in esters (1.35 Å) or thio esters (1.75 Å). In addition, the C(carbonyl)–X–C bond angle is larger (120°) in peptides than in thio esters (99.4°) and esters (116°). Thus, interactions with both active site residues and at the subsites could be significantly altered by changing a peptide bond to a thio ester or ester. Alternately, the kinetic differences between peptides and esters could be due not so much to different binding modes but to differences in rate-limiting steps in the catalytic mechanism. For example, the rate of product dissociation may be mechanistically significant and could account for the peptide vs. ester and thio ester difference (Vallee, 1983). Faster dissociation of the amine product compared to the corresponding alcohol or thiol would also explain the rate differences that we observed.

Inhibitors. The best synthetic metalloprotease inhibitors that have thus far been reported have a peptide backbone that is recognized by the enzyme, attached to functional groups such as hydroxamic acids, thiols, or carboxyl groups, which can act as ligands for the active site zinc (Holmquist & Vallee, 1979; Cushman et al., 1977; Nishino & Powers, 1978). Hydroxamic acid peptides with sequences specific for *C. histolyticum* collagenase proved to be only moderate inhibitors with the best peptide hydroxamic acid having a K_I of 0.24 mM. The K_I values for analogous peptide hydroxamic acids developed for use with thermolysin (Ninshino & Powers, 1978) and *Pseudomonas aeruginosa* elastase (Nishino & Powers, 1980) were roughly 3 orders of magnitude better reversible inhibitors with the best K_I values being 0.30 μM and 0.044 μM, respectively. In order to take advantage of subsite binding on both sides of the scissile bond, we synthesized three unique *N*-hydroxy peptides containing a hydroxamic acid functional group in the center of the peptide sequence. Unfortunately, two were not inhibitors, and one was a very poor inhibitor. The lack of inhibition of these compounds is probably due to their inability to ionize to a stabilized anion, a pathway available to N-unsubstituted hydroxamic acids that probably accounts for potent inhibition of thermolysin by these compounds (Holmes & Matthews, 1981).

Mercaptan peptides were again only moderately good inhibitors for *C. histolyticum* collagenase with the best inhibitor having a K_I value of 0.02 mM. Mercaptan-containing peptides have been used to inhibit angiotensin converting enzyme (Cushman et al., 1977), carboxypeptidase A (Ondetti et al., 1979), thermolysin (Nishino & Powers, 1979), and *P. aeruginosa* elastase (Nishino & Powers, 1980) with the best K_I values being 1.7 nM, 11 nM, 750 nM and 64 nM, respectively. Carboxymethyl peptides have been shown to be excellent inhibitors of angiotensin converting enzyme with IC_{50} values as low as 1.2 nM (Patchett et al., 1980), but again, the one carboxymethyl inhibitor for collagenase that we synthesized only had a K_I of 1.2 mM.

Haloacetyl peptides have been successfully used to inhibit carboxypeptidase A and B (Hass & Neurath, 1971; Plummer, 1971), and *N*-chloroacetyl-*N*-hydroxy peptides and amino acid derivatives have been shown to be irreversible inhibitors of thermolysin and *P. aeruginosa* elastase (Rasnick & Powers, 1978; Nishino & Powers, 1980). However, none of the haloacetyl peptides or acryloyl peptides that we designed for *C. histolyticum* collagenase inhibited the enzyme.

Thus far, phosphoryl peptides are the best inhibitors for *C. histolyticum* collagenase with the best reported IC_{50} value being 14 μM (Galardy & Grobelny, 1983). This inhibitor utilized P_1–P_3' interactions. However, the collagenase phosphoryl peptides were once again ca. 1000 times less potent than the phosphoryl inhibitors reported for other metalloproteases such as thermolysin and angiotensin converting enzyme (Kam et al., 1979; Galardy & Grobelny, 1983).

It is apparent now that short peptide sequences with attached metal chelating ligands are not good inhibitors for collagenase. In the future it will be necessary to utilize longer sequences to obtain interaction with more recognition sites or to design sequences or structures that imitate the geometry of the collagen triple helix.

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Supplementary Material Available

Syntheses of the new compounds reported in this paper and three tables depicting the effect of 4,4′-dithiodipyridine on hydrolysis of thio esters, effect of organic solvents on hydrolysis rates of thio esters by collagenase, and reaction of irreversible inhibitors with collagenase (31 pages). Ordering information is given on any current masthead page.

Registry No. $HSCH_2CH_2CO$-Pro-Nba, 96166-25-5; Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba, 96166-26-6; Boc-Abz-Gly-Pro-Leu-Gly-Pro-Nba, 96166-27-7; Z-Gly-Pro-Leu-Gly-Pro-NH_2, 96166-28-8; Z-Gly-Pro-Leu-SCH_2CO-Pro-NH_2, 96166-29-9; Z-Gly-Pro-Leu-OCH_2CO-Pro-NH_2, 96194-13-7; Z-Gly-Pro-Leu-Gly-Pro-Nba, 96166-30-2; Z-Gly-Pro-Leu-SCH_2CO-Pro-Nba, 96166-31-3; Boc-Abz-Gly-Pro-Leu-SCH_2CO-Pro-Nba, 96194-14-8; Abz-Gly-Pro-Leu-Gly-Pro-Nba, 96166-32-4; FA-Leu-Gly-Pro-Ala-OH, 96194-15-9; Ac-SCH_2CO-Pro-Nba, 96166-33-5; Ac-SCH_2CH_2CO-Pro-Nba, 96166-34-6; Z-Gly-Pro-Leu-SCH_3, 96166-35-7; Ac-Leu-SCH_2CO-Pro-Nba, 96166-36-8; Abz-Pro-Leu-Gly-Pro-Nba, 96166-37-9; Boc-Gly-Pro-Leu-(N-OH)Gly-OEt, 96166-38-0; H-Gly-Pro-Leu-(N-OH)Gly-Pro-OH, 96194-16-0; $HOCH_2CO$-Pro-NH_2, 96166-39-1; Z-Gly-Pro-Leu-NHOH, 96166-40-4; $HONHCOCH_2CO$-Pro-NH_2, 96194-17-1; $HONHCOCH_2CH_2CO$-Pro-NH_2, 96194-18-2; Boc-Gly-Pro-Leu-(N-OH)Gly-Pro-OH, 96194-19-3; Boc-Gly-Pro-OBzl, 29776-78-1; Boc-Gly-Pro-NHBzl, 96166-41-5; $HO_2CCH_2NHCH_2CO$-Pro-Nba, 96194-20-6; $HSCH_2CH_2CO$-Pro-NH_2, 96166-42-6; $HSCH_2CH_2CO$-Pro-NHBzl, 96166-43-7; Z-Gly-Pro-Leu-OH, 2646-63-1; Z-Gly-Leu-NHOH, 66179-51-9; Z-Gly-Gly-Leu-NHOH, 66179-56-4; HONHCOCH(i-Bu)CO-Ala-Gly-NH_2, 71431-46-4; Pro-Nba·HCl, 96194-12-6; Boc-Abz-Gly-Pro-OH, 96166-44-8; Leu-SCH_2CO-Pro-Nba·HCl, 96166-45-9; Boc-Abz-OH, 68790-38-5; Gly-Pro-Leu-Gly-Pro-Nba·HCl, 96166-46-0; 3-(acetylmercapto)propionic acid, 41345-70-4; collagenase, 9001-12-1.

References

Balaevskaya, T., Solovieva, N. J., & Orekhovich, V. N. (1981) *Bioorg. Khim. 7*, 1553.

Bond, M. D., & Van Wart, H. E. (1984a) *Biochemistry 23*, 3077.

Bond, M. D., & Van Wart, H. E. (1984b) *Biochemistry 23*, 3085.

Carmel, A., Zur, M., Yaron, A., & Katohalski, E. (1973) *FEBS Lett. 30*, 11.

Castillo, M. J., Kurachi, K., Nishino, N., Ohkuba, I., & Powers, J. C. (1983) *Biochemistry 22*, 1021.
Cushman, D. W., Cheung, H. S., Sabo, E. F., & Ondetti, M. A. (1977) *Biochemistry 16*, 5485.
Galardy, R. E., & Grobelny, P. (1983) *Biochemistry 22*, 4556.
Grassetti, D. R., & Murray, J. F. (1967) *Arch. Biochem. Biophys. 119*, 41.
Grassman, W., & Nordwig, A. (1960) *Hoppe-Seyler's Z. Physiol. Chem. 322*, 267.
Grassman, W.,'Nordwig, A., & Hermann, H. (1961) *Hoppe-Seyler's Z. Physiol. Chemie 323*, 48.
Hanes, C. S. (1932) *Biochem. J. 26*, 1406.
Harper, J. W., Cook, R. R., Roberts, L. J., McLaughlin, B. J., & Powers, J. C. (1984) *Biochemistry 23*, 2995.
Hass, F. M., & Neurath, H. (1971) *Biochemistry 10*, 3541.
Holmes, M. A., & Matthews, B. W. (1981) *Biochemistry 20*, 6912.
Holmquist, B., & Vallee, B. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6216.
Kam, C. H., Nishino, H., & Powers, J. C. (1979) *Biochemistry 18*, 3032.
Lapiere, C. M., & Gross, J. (1963) in *Mechanisms of Hard Tissue Destruction* (Sognnaes, R., Ed.) pp 663–694, American Association of Advances in Science, Washington, DC.
Latt, S. A., Auld, D. S., & Vallee, B. L. (1972) *Anal. Biochem. 50*, 56.
Lwebuga-Mukasa, J. S., Harper, E., & Taylor, P. (1976) *Biochemistry 15*, 4736.
Mandl, I., Zipper, H., & Ferguson, L. T. (1958) *Arch. Biochem. Biophys. 74*, 465.
McRae, B. J., Kurachi, K., Heinmark, R. L., Fujikawa, K., Davie, E. W., & Powers, J. C. (1981) *Biochemistry 20*, 7196.
Metrione, R. M. (1972) *Biochim. Biophys. Acta 268*, 518.
Nagai, Y., Sakakibara, S., Noda, H., & Akabori, S. (1960) *Biochim. Biophys. Acta 37*, 56.
Nishino, N., & Powers, J. C. (1978) *Biochemistry 17*, 2846.
Nishino, N., & Powers, J. C. (1979) *Biochemistry 18*, 4340.
Nishino, N., & Powers, J. C. (1980) *J. Biol. Chem. 255*, 3482.
Ondetti, M. A., Condon, M. E., Reid, J., Sabo, E. F., Cheung, H. S., & Cushman, D. W. (1979) *Biochemistry 18*, 1427.
Patchett, A. A., Harris, E., Tristram, E. W., Wyvratt, M. J., Wu, M. T., Taub, D., Peterson, E. R., Ikeler, J. J, ten Bioeke, J., Payne, L. G., Ondeyka, D. L., Thossett, E. D., Greenlee, W. J., Lohr, W. S., Hoffsommer, R. D., Joshua, H., Ruyle, W. V., Rothrock, J. W., Aster, S. D., Maycock, A. L., Robinson, F. M., Hirschmann, R., Sweet, C. S., Ulm, E. H., Gross, D. M., Vassil, T. C., & Stone, C. A. (1980) *Nature (London) 288*, 280.
Persson, A., & Wilson, I. B. (1977) *Anal. Biochem. 83*, 296.
Plummer, T. H., Jr., (1971) *J. Biol. Chem. 246*, 2930.
Powers, J. C. (1977) in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins* (Weinstein, B., Ed.) Vol. 4, pp 65–178, Dekker, New York, NY.
Rasnick, D., & Powers, J. C. (1978) *Biochemistry 17*, 4363.
Robertson, P. B., Taylor, R. E., & Fullmer, H. M. (1972) *Clin. Chim. Acta 42*, 43.
Rosen, H. (1957) *Arch. Biochem. Biophys. 67*, 10.
Sakamoto, S., Goldhaber, P., & Glimcher, M.J. (1972) *Proc. Soc. Exp. Biol. Med. 139*, 1057.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157.
Seifter, S., & Gallop, P. (1962) *Methods Enzymol. 5*, 659.
Seifter, S., & Harper, F. (1971) *Enzymes, 3rd Ed. 3*, 649.
Soberano, M. E., & Schoellmann, G. (1972) *Biochim. Biophys. Acta 271*, 133.
Stevens, F. S., Torre-Blanco, A., & Hunter, J. A. A. (1975) *Biochim. Biophys. Acta 405*, 188.
Suh, J., & Kaiser, E. T. (1975) *Biochem. Biophys. Res. Commun. 64*, 863.
Vallee, B. L., Galdes, A., Auld, D. S., & Riordau, J. F. (1983) in *Zinc Enzymes* (Spiro, T. G., Ed.) pp 25–75, Wiley-Interscience, New York.
Van Wart, H. E., & Steinbrink, D. R. (1981) *Anal. Biochem. 113*, 356.
Wunsch, E., & Heidrich, H. (1963) *Hoppe-Seyler's Z. Physiol. Chem. 333*, 149.
Yagisawa, S., Morita, F., Nagai, Y., Noda, A., & Ogura, Y. (1965) *J. Biochem. (Tokyo) 58*, 407.
Zachrias, D. E., Murray-Rust, P., Preston, R. M., & Glusker, J. P. (1983) *Arch. Biochem. Biophys. 222*, 22.