Fantoni, A., De la Chapelle, A., Rifkind, R. A., & Marks, P. A. (1968) *J. Mol. Biol. 83*, 79–91.
Franklin, S. G., & Zweidler, A. (1977) *Nature (London) 266*, 273–275.
Gilman, J. G. (1972) *Ann. N.Y. Acad. Sci. 416*, 273–275.
Gilman, J. G. (1976) *Biochem. J. 155*, 231–241.
Hunt, L. T., & Dayhoff, M. O. (1976) in *Atlas of Protein Sequence and Structure* (Dayhoff, M. O., Ed.), Vol. 5, Suppl. 2, pp 191–223, National Biochemical Research Foundation, Washington, D.C.
Ikawa, Y., Soma, G-I., & Obinata, M. (1979) *Proc. Jpn. Acad., Ser. B 55*, 311–316.
Kameji, R., Obinata, M., Natori, Y., & Ikawa, Y. (1977) *J. Biochem. (Tokyo) 51*, 1901–1920.
Kovach, J. S., Marks, P. A., Russell, E. S., & Epler, H. (1967) *J. Mol. Biol. 25*, 131–142.
Lanyon, W. G., Ottolenghi, S., & Williamson, R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 253–262.
Lawn, R. M., Fritch, E. F., Parker, R. C., Blake, G., & Maniatis, T. (1978) *Cell 15*, 1157–1174.
Monahan, J. J., Woo, S. L. O., Liarakos, C. D., & O'Malley, B. W. (1977) *J. Biol. Chem. 252*, 4722–4728.
Monte, M., Beuzard, Y., & Rosa, J. (1976) *Ann. J. Clin. Pathol. 66*, 753–759.
Pelham, R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247–259.
Rovera, G., Abramezuk, J, & Surrey, S. (1977) *FEBS Lett. 81*, 366–369.
Ryffel, G. U., & McCarthy, B. J. (1975) *Biochemistry 14*, 1378–1385.
Schroeder, W. A., Huisman, T. H. J., Shelton, R., Shelton, J. B., Kleinhauer, E. F., Dozy, A. M., & Robberson, B. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 537–563.
Terada, M., Bank, J., & Marks, P. A. (1971) *J. Mol. Biol. 62*, 347–360.
Tuan, D., Biro, P. A., de Riel, J. K., Lazarus, H., & Forget, B. G. (1979) *Nucleic Acids Res. 6*, 2519–2544.
Wilson, H. T., Wilson, L. B., de Riel, J. K., Villa-Kemaroff, L., Efstratiadis, A., Forget, B. G., & Weissman, S. M. (1978) *Nucleic Acids Res. 5*, 563–581.

Studies on Reactivity of Human Leukocyte Elastase, Cathepsin G, and Porcine Pancreatic Elastase toward Peptides Including Sequences Related to the Reactive Site of α_1-Protease Inhibitor (α_1-Antitrypsin)†

Brian McRae, Kiichiro Nakajima, James Travis, and James C. Powers*

ABSTRACT: The reaction of human leukocyte (HL) elastase, porcine pancreatic (PP) elastase, and human cathepsin G has been studied with peptide substrates. Most of the peptides had a prolyl residue at P_2, since several serine proteases have been shown to productively bind such substrates only with the proline at the subsite (S_2) adjacent to the primary substrate binding site (S_1). HL elastase prefers a valyl residue over Ala or Met at S_1 while PP elastase prefers an Ala. With both elastases, extension of the peptide chain results in significant increases in k_{cat}/K_M. With the appropriate substrates, HL elastase is as reactive as PP elastase. Cathepsin G shows a preference for Phe over Met at S_1 and a preference for Phe over Ala or Leu at S_1'. Extension of the peptide chain yields little increase in rate, and thus the k_{cat}/K_M values observed with cathepsin G are not as large as those of the other enzymes. The α_1-protease inhibitor (α_1-PI) reactive site has recently been shown to have the sequence -Ala-Ile-Pro-Met*Ser-Ile-Pro-Pro-, where the asterisk indicates the bond cleaved (P_1–P_1') when α_1-PI·protease complexes are split. The octapeptide Ac-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro-NH_2 and the analogue with a P_1' Thr instead of Ser were synthesized. All three enzymes bound to and cleaved the peptides at the P_1 Met bond. The K_M values were in the millimolar range, showing that this particular sequence alone does not account for the tight binding of serine proteases to α_1-PI. The k_{cat} values, a measure of the ease with which certain types of bond formation between proteases and α_1-PI would occur, were higher for the P_1' Ser octapeptide than for the Thr analogue, indicating that relatively minor amino acid substitutions in the α_1-PI reactive site will profoundly influence its reactivity toward various proteases. Inactivation of α_1-PI in the lung by oxidation and the resulting protease imbalance is the currently accepted model for the development of emphysema. In the majority of cases studied, oxidation of the P_1 Met residue of simple peptides to the sulfoxide resulted in decreased binding to the enzymes studied, and a decreased k_{cat}/K_M. Reduction of substrate effectiveness was greatest with HL elastase for the P_1' Ser peptides compared to the P_1' Thr peptides. Reactive site substitutions could effect the degree to which oxidation is damaging to the inhibitor and may be one possible explanation for the greater susceptibility to emphysema of some individuals with normal α_1-PI.

The granule fraction of human leukocytes contains major amounts of two serine proteases, HL[1] elastase and cathepsin G. HL elastase has been implicated in the proteolysis of lung elastin which is observed in pulmonary emphysema (Mittman, 1972; Turino et al., 1974; Hance & Crystal, 1975). Cathepsin G is also capable of attacking elastin and other connective tissue components. Granulocyte serine proteases are normally involved in the turnover of dead lung tissue and in the destruction of invading bacteria (Blondin et al., 1978) and only

† From the School of Chemistry, Georgia Institute of Technology, Atlanta, Georgia 30332 (B.M., K.N., and J.C.P.), and Department of Biochemistry, University of Georgia, Athens, Georgia 30602 (J.T.). *Received February 21, 1980.* This research was supported by a grant from the Council for Tobacco Research.

[1] Abbreviations used: Aala, $-NHN(CH_3)CO-$; MeO-Suc, methoxysuccinyl; Suc, succinyl; α_1-PI, α_1-protease inhibitor (α_1-antitrypsin); PP, porcine pancreatic; HL, human leukocyte; NA, 4-nitroanilide.

0006-2960/80/0419-3973$01.00/0 © 1980 American Chemical Society

Table I: Hydrolysis of Peptide Substrates by Human Leukocyte Elastase[a]

substrate P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'	K_M[b] (mM)	k_{cat}[b] (s^{-1})	k_{cat}/K_M[b] (M^{-1} s^{-1})
Ac-Ala-Ala-Pro-Ala-NH_2	12 (3)	0.33 (0.05)	28
Ac-Ala-Ala-Pro-Val-NH_2	6.0 (2)	2.4 (0.3)	400
Ac-Ala-Ala-Ala-Val-NH_2	[c]		
MeO-Suc-Ala-Ala-Pro-Val-NH_2	4.4 (0.4)	3.9 (0.4)	910
Ac-Ala-Ala-Pro-Val-Ala-Ala-NH_2	0.74 (0.03)	34 (0.5)	46000
Ac-Ala-Ala-Pro-Val-Thr-Ala-NH_2	0.82 (0.05)	38 (3)	46000
Ac-Ala-Ala-Pro-Met-Ser-Ala-NH_2	2.6 (0.2)	2.1 (0.2)	850
Ac-Ala-Ala-Pro-Met(O)-Ser-Ala-NH_2	5.4 (0.2)	0.86 (0.02)	157
Ac-Ala-Ala-Pro-Met-Thr-Ala-NH_2	2.7 (0.2)	13 (0.6)	4700
Ac-Ala-Ala-Pro-Met(O)-Thr-Ala-NH_2	6.9 (0.05)	13 (0.1)	1900
Boc-Ala-Ala-Pro-Met-Thr-Ala-NH_2	2.7 (0.4)	14 (4)	5000
Ac-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro-NH_2	1.6 (0.1)	16 (0.9)	10000
Ac-Ala-Ile-Pro-Met(O)-Ser-Ile-Pro-Pro-NH_2	3.6 (0.2)	0.094 (0.005)	27
Ac-Ala-Ile-Pro-Met-Thr-Ile-Pro-Pro-NH_2	1.9 (0.2)	4.1 (0.4)	2300
Ac-Ala-Ile-Pro-Met(O)-Thr-Ile-Pro-Pro-NH_2	3.2 (0.3)	0.09 (0.02)	29

[a] pH 7.5; 0.040 M phosphate; 0.50 M NaCl; 37 °C. [b] Standard deviations are reported in parentheses. [c] Insoluble.

become damaging when released from leukocytes.

The plasma of healthy individuals contains large amounts of protease inhibitors including α_1-protease inhibitor (α_1-PI) which inhibits both HL and pancreatic elastase and cathepsin G. The inhibition of serine proteases by α-PI involves formation of a stable complex between α_1-PI and the enzyme. The sequence at the reactive site of α_1-PI has been shown to be -Ala-Ile-Pro-Met*Ser-Ile-Pro-Pro-, where the asterisk indicates the site of cleavage in modified α_1-PI (Johnson & Travis, 1978). Oxidation of the P_1 Met residue[2] of α_1-PI to the sulfoxide has been shown to destroy its inhibitory capacity toward most serine proteases (Johnson & Travis, 1979).

The active sites of HL elastase and cathepsin G have been studied with peptide 4-nitroanilides (Nakajima et al., 1978; Zimmerman & Ashe, 1977), peptide chloromethyl ketones (Powers et al., 1977), and azapeptides (Powers et al., 1978). No studies with simple peptides have been reported. In this paper we report a study of the reaction of HL elastase, cathepsin G, and porcine pancreatic elastase with peptides containing amino acid residues not only on the carbonyl side of the bond cleaved (P residues) but also on the imino side (P′ residues). In addition, we have synthesized a number of peptides whose sequence is identical with that of various sections of the α_1-PI reactive site and show how changes in their state of oxidation and sequence affect their reactivity toward HL elastase, cathepsin G, and porcine pancreatic elastase. The results should be useful not only for the design of more specific and reactive inhibitors of leukocyte proteases but also for understanding how α_1-PI interacts with various proteases.

Materials and Methods

Porcine pancreatic elastase (6 times crystallized) was purchased from Worthington Biochemical Corp., Freehold, NJ, and was assayed by using Boc-Ala-ONp (Visser & Blout, 1972) and titrated with Ac-Ala-Ala-Aala-ONp (Powers & Gupton, 1977). Human granulocyte elastase and cathepsin G were generously supplied by Dr. J. Travis and his group of the University of Georgia, Athens, Ga. Human granulocyte elastase was titrated with Ac-Ala-Ala-Aala-ONp (Powers & Gupton, 1977), and the cathepsin G was assayed by measuring the rate of hydrolysis of Boc-Tyr-ONp (Powers et al., 1977). All common chemicals and solvents used were reagent grade.

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

Experimental details for the synthesis of the new compounds can be found in the supplementary material (see paragraph at end of paper regarding supplementary material).

Kinetic Measurements. The enzymatic hydrolysis of peptides and peptide amides was measured by using a modification of the ninhydrin colorimetric analysis for amino acids (Rosen, 1957). Reactions were conducted in 40 mM phosphate at pH 7.50 and 37 °C. For human granulocyte elastase and cathepsin G, 0.50 M NaCl was also present. Following the addition of the enzyme to the reaction mixture, aliquots of 0.25 mL were taken periodically and quenched with 0.25 mL of 5% trichloroacetic acid. Following the procedure used for color development, the A_{570} values were measured and plotted vs. time to obtain the reaction rates. Rates were converted to molar quantities by means of a standard curve. The ninhydrin colorimetric procedure was performed with known concentrations of the hydrolysis product for each substrate under identical reaction conditions, and the ϵ value was obtained from a plot of A_{570} vs. concentration. Because the ϵ values slowly decreased due to ninhydrin oxidation, the procedure was repeated every few days. Strict Michaelis–Menten kinetics were observed, and values for K_M, k_{cat}, and standard deviations were obtained from Lineweaver–Burk plots by using a linear regression program.

Determination of the Cleavage Site. Thin-layer electrophoresis was carried out to determine the bond split by the enzyme. The reaction mixtures were spotted on silica gel plates (5 × 20 cm) and subjected to electrophoresis at 1000 V for 30 min, using an aqueous buffer containing 2.5% formic acid and 7.8% acetic acid (pH 1.85). All ninhydrin-positive products were identified by spraying with 0.3% ninhydrin–acetone and heating at 120 °C for 15 min. The plates were then sprayed with concentrated sulfuric acid and heated on a hot plate to identify all products. In all cases, the expected bond was split without any trace of other bond cleavages.

Results

The kinetic parameters for the hydrolysis of a number of peptide substrates by HL elastase, porcine pancreatic elastase, and cathepsin G are reported respectively in Tables I, II, and III. The rates were followed by means of a ninhydrin assay using in each case the hydrolysis product as the standard.

The cleavage site of all the peptides except the octapeptides was investigated by thin-layer electrophoresis. In the electrophoresis experiments, the conditions were adjusted in each case until the R_f value of the ninhydrin-positive hydrolysis product had a value of 0.6 to 0.7. Under these conditions the

Table II: Hydrolysis of Peptide Substrates by Porcine Pancreatic Elastase[a]

substrate P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'	K_M[b] (mM)	k_{cat}[b] (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Ac-Ala-Ala-Pro-Ala-NH_2	2.0 (0.2)	5.2 (0.4)	2700
Ac-Ala-Ala-Pro-Val-NH_2	5.5 (0.8)	1.4 (0.2)	240
MeO-Suc-Ala-Ala-Pro-Val-NH_2	5.6 (0.8)	2.0 (0.3)	350
Ac-Ala-Ala-Ala-Val-NH_2	[c]		
Ac-Ala-Ala-Pro-Val-Ala-Ala-NH_2	0.92 (0.06)	38 (1)	41000
Ac-Ala-Ala-Pro-Val-Thr-Ala-NH_2	0.74 (0.04)	53 (13)	71000
Ac-Ala-Ala-Pro-Met-Ser-Ala-NH_2	3.4 (0.4)	3.7 (0.3)	1200
Ac-Ala-Ala-Pro-Met(O)-Ser-Ala-NH_2	15 (4)	1.5 (0.4)	96
Boc-Ala-Ala-Pro-Met-Thr-Ala-NH_2	3.4 (0.3)	9.6 (1.1)	2800
Ac-Ala-Ala-Pro-Met-Thr-Ala-NH_2	2.9 (0.2)	8.4 (0.6)	3000
Ac-Ala-Ala-Pro-Met(O)-Thr-Ala-NH_2	12 (0.8)	5.0 (0.3)	420
Ac-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro-NH_2	0.55 (0.01)	5.7 (0.03)	11000
Ac-Ala-Ile-Pro-Met(O)-Ser-Ile-Pro-Pro-NH_2	10 (1)	1.6 (0.2)	160
Ac-Ala-Ile-Pro-Met-Thr-Ile-Pro-Pro-NH_2	4.0 (0.1)	1.5 (0.4)	370
Ac-Ala-Ile-Pro-Met(O)-Thr-Ile-Pro-Pro-NH_2	10 (5)	0.4 (0.2)	38

[a] pH 7.5; 0.040 M phosphate; 37 °C. [b] Standard deviations are reported in parentheses. [c] Insoluble.

Table III: Hydrolysis of Peptide Substrates by Cathepsin G[a]

substrate P_4 P_3 P_2 P_1 P_1' P_2' P_3' P_4'	K_M[c] (mM)	k_{cat}[c] (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
Ac-Ala-Ala-Ala-Phe-NH_2	[d]		
Ac-Ala-Ala-Pro-Phe-NH_2	9.0 (1.6)	0.50 (0.08)	56
MeO-Suc-Ala-Ala-Pro-Phe-NH_2	4.7 (0.8)	0.32 (0.02)	69
Suc-Ala-Ala-Pro-Phe-Ala-NH_2	10 (1)	13 (2)	1300
Suc-Ala-Ala-Pro-Phe-Phe-NH_2	1.7 (0.5)	3.2 (1.1)	1900
Suc-Ala-Ala-Pro-Phe-Leu-NH_2	3.4 (0.6)	2.7 (0.4)	780
Ac-Ala-Ala-Pro-Phe-Ala-Ala-NH_2[b]	12 (9)	12 (9)	1000
Ac-Ala-Ala-Pro-Phe-Thr-Ala-NH_2	[d]		
Ac-Ala-Ala-Pro-Val-Ala-Ala-NH_2	2.0 (1)	0.025 (0.007)	10
Ac-Ala-Ala-Pro-Met-Ser-Ala-NH_2	4.5 (0.1)	0.48 (0.01)	110
Ac-Ala-Ala-Pro-Met(O)-Ser-Ala-NH_2	19 (2)	0.12 (0.01)	7
Ac-Ala-Ala-Pro-Met-Thr-Ala-NH_2	2.2 (0.2)	0.8 (0.3)	350
Ac-Ala-Ala-Pro-Met(O)-Thr-Ala-NH_2	7 (3)	0.01 (0.01)	1.5
Boc-Ala-Ala-Pro-Met-Thr-Ala-NH_2	2.1 (0.2)	0.7 (0.3)	320
Ac-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro-NH_2	4.6 (0.9)	4.1 (0.8)	900
Ac-Ala-Ile-Pro-Met(O)-Ser-Ile-Pro-Pro-NH_2	3.6 (0.3)	0.14 (0.01)	39
Ac-Ala-Ile-Pro-Met-Thr-Ile-Pro-Pro-NH_2	3.6 (0.1)	1.3 (0.05)	390
Ac-Ala-Ile-Pro-Met(O)-Thr-Ile-Pro-Pro-NH_2	7.2 (0.7)	0.42 (0.07)	69

[a] pH 7.50; 0.040 M phosphate; 0.50 M NaCl; 37 °C. [b] Reaction mixture contained 10% Me_2SO. [c] Standard deviations are reported in parentheses. [d] Insoluble.

R_f value of the carboxylic acid product was ~0.1. In each case, the R_f value of the ninhydrin-positive hydrolysis product corresponded to that of an authentic sample of the expected product, and no other ninhydrin-positive spots were detected. With the tetrapeptides, no ninhydrin-positive spots were detected. In the case of the octapeptides, the hydrolysis mixture was investigated by TLC using silica gel G plates and development with butanol–acetic acid–water (4:1:1) or butanol–acetic acid–pyridine–water (4:1:2:1). In this case, the R_f value of the ninhydrin hydrolysis product corresponded to the R_f of the authentic tetrapeptide expected, and no other products were detected.

Discussion

The subsite specificity of HL elastase and cathepsin G has been studied previously by using chloromethyl ketone inhibitors (Powers et al., 1977; Tuhy & Powers, 1975) and peptide 4-nitroanilide substrates (Nakajima et al., 1978; Zimmerman & Ashe, 1977). Porcine pancreatic elastase has been more widely investigated. Kinetic results have been reported with peptide substrates (Bauer et al., 1976; Thompson & Blout, 1973b,c), peptide 4-nitroanilides (Nakajima et al., 1978; Bieth & Wermuth, 1973; Zimmerman & Ashe, 1977), and peptide chloromethyl ketone inhibitors (Powers & Tuhy, 1973; Thompson & Blout, 1973a; Thomson & Denniss, 1973). All three enzymes are inhibited by the plasma protease inhibitor α_1-protease inhibitor. Both elastases will induce emphysema in experimental animals, with the porcine pancreatic enzyme being more effective (Senior et al., 1977).

One goal of this research was to study the subsite specificity of HL elastase and cathepsin G and to compare the reactivities of HL elastase and PP elastase. Simple peptide substrates were considered to be the ideal vehicle for the investigation, since with serine proteases it has been long established that K_M values are equal to the true dissociation constants (K_s) of the E·S complex and k_{cat} is equal to the rate-limiting acylation step (k_2). However the possibility of nonproductive binding must be considered before this can be accepted with confidence.

Proline residues have been shown to restrict the binding modes of substrates to serine proteases. Bovine chymotrypsin A_γ (Segal, 1972) and porcine pancreatic elastase (Thompson & Blout, 1973b) both prefer to bind proline residues at the S_2 subsite. Therefore, the majority of the substrates we synthesized contained a proline residue (P_2) adjacent to the P_1 residue. The presence of such a proline residue is indeed sufficient to restrict the binding mode to cathepsin G since we have recently shown that cleavage of Suc-Ala-Pro-Leu-Phe-NA takes place at the Leu-Phe bond instead of at the Phe-NA bond, despite the fact that cathepsin G prefers P_1 Phe

residues (Nakajima et al., 1978). The same cannot be stated unequivocally for HL elastase, since tetrapeptide chloromethyl ketones with either a P_3 or P_2 prolyl residue will effectively inhibit the enzyme (Tuhy & Powers, 1975). Examination of the reaction mixtures for each substrate with each enzyme was carried out by electrophoresis or TLC, and in no case were we able to detect cleavage products resulting from cleavage at any bonds other than the one indicated. Thus, we are confident that a P_2 prolyl residue prevents alternative productive binding modes in each case. These results do not rule out nonproductive binding modes, however. If such exist, K_M and k_{cat} no longer represent true binding or catalytic constants, respectively, but would be $k_{cat} = k_2/(1 + K_s/K_s')$ and $K_M = K_s/(1 + K_s/K_s')$ where K_s' represents the dissociation constant for nonproductive binding modes. K_M and k_{cat} would then only represent lower limits for the true kinetic parameters, K_s and k_{cat}. However, it can be seen that k_{cat}/K_M is still equal to k_2/K_s, and thus k_{cat}/K_M is a valid measure even with the existence of nonproductive binding modes.

Human Leukocyte Elastase. HL elastase prefers a P_1 valyl residue over an alanyl residue. This effect has been observed earlier with chloromethyl ketone inhibitors (Powers et al., 1977) and 4-nitroanilide substrates (Zimmerman & Ashe, 1977). The primary effect is a sevenfold increase in k_{cat}, while K_M decreases by twofold (comparison of Ac-Ala-Ala-Pro-Ala-NH_2 with Ac-Ala-Ala-Pro-Val-NH_2). A P_1 Val is also preferred over Met. Ac-Ala-Ala-Pro-Val*Thr-Ala-NH_2 (asterick indicates cleavage site) binds threefold more tightly than Ac-Ala-Ala-Pro-Met*Thr-Ala-NH_2 and has a threefold higher k_{cat} value.

Changing the P_5 group of a peptide substrate from acetyl to methoxysuccinyl (MeO-Suc) or the hydrophobic Boc group has almost no effect on substrate hydrolysis. This is in contrast to the peptide chloromethyl ketone inhibition reaction where a P_5 MeO-Suc group significantly increases the rate of inhibition (Powers et al., 1977). This tends to indicate that P_5–S_5 interactions do not affect binding to the enzyme but have a significant influence on the bond-forming reaction with chloromethyl ketones (nucleophilic substitution) which differs from that involved in substrate hydrolysis (acylation).

Extension of the peptide chain to encompass P_1' and P_2' residues results in over a 7-fold decrease in K_M and a 14-fold increase in k_{cat}. Comparison of the hexapeptides with octapeptides in the series of compounds with a P_1 Met residue shows that extension of the peptide chain to include P_3' and P_4' prolyl residues results in a slight improvement of many of the kinetic parameters. These results are the first indication that HL elastase has an extended substrate binding region on both sides of the scissile bond. There is certainly recognition to at least P_5 on the P side and to P_3' and possibly further on the P′ side of the scissile bond. This demonstrates that the extended substrate binding site of HL elastase encompasses at least seven or eight amino acid residues and has an equal number of subsites.

The S_1' subsite of HL elastase prefers an Ala or Thr residue over a Ser in the case of the hexapeptide substrates. The primary effect is in k_{cat}; the K_M values for the two P_1 valine hexapeptides are approximately the same as are the K_M values for the three P_1 methionine hexapeptides. However, the k_{cat} for Ac-Ala-Ala-Pro-Met*Thr-Ala-NH_2 is 6 times larger than that for Ac-Ala-Ala-Pro-Met*Ser-Ala-NH_2. With the octapeptides (Met), the K_M values are again very similar. However, in this case the k_{cat} for the P_1' Ser octapeptide is fourfold higher than that for the P_1' Thr octapeptide. Evidently k_{cat} is highly dependent not only on the nature of the P_1' residue but also on the geometry of the residue in the E·S complex which would be affected by interactions at other P′ subsites.

Porcine Pancreatic Elastase. The subsite specifity of porcine pancreatic elastase has been widely studied with both substrates and inhibitors. Analysis of reported cleavages of peptides by porcine pancreatic elastase indicates that the enzyme most frequently cleaves after Ile (probability = 0.42), Val (0.39), and Ala (0.35) residues (Powers et al., 1977). However, with tetrapeptide substrates the P_1 Ala amide binds slightly better and has a slightly higher k_{cat} than the P_1 Val amide.

Extension of the peptide chain to encompass P_1' and P_2' residues increases k_{cat}/K_M by a factor of 170–300. This is partially due to a six- to sevenfold lower K_M. But the major effect is a 27–38-fold increase in k_{cat}. With the hexapeptides with a P_1 Val, a P_1' Thr is slightly preferred over Ala. In the series of compounds with a P_1 Met residue, extension of the chain beyond P_2' results in an increase in k_{cat}/K_M in one case (P_1' = Ser) and a decrease in another (P_1' = Thr).

Comparison of Human Leukocyte and Porcine Pancreatic Elastase. HL elastase has a larger S_1 subsite than PP elastase and prefers a P_1 Val rather than Ala. This observation confirms similar results obtained with peptide chloromethyl ketone inhibitors (Powers et al., 1977) and 4-nitroanilide substrates (Zimmerman & Ashe, 1977).

HL elastase, with the appropriate peptide substrate, is nearly as good an enzyme as porcine pancreatic elastase. Previous studies have indicated that PP elastase would attack elastin more readily and would hydrolyze certain substrates such as Boc-Ala-ONp or Suc-Ala-Ala-Ala-NA more rapidly than HL elastase. In addition, PP elastase induced emphysema more readily in experimental animals (Senior et al., 1977). It is now evident that this difference is only due to an inappropriate substrate sequence. Our data show that the maximum k_{cat}/K_M values obtained with HL elastase and good HL elastase substrates are essentially the same as k_{cat}/K_M values obtained with PP elastase and good PP elastase substrates. In the case of HL elastase, the k_{cat}/K_M reaches a maximum of 46 000 M^{-1} s^{-1} with the hexapeptides containing a P_1 Val. With PP elastase, the maximum k_{cat}/K_M with hexapeptides (P_1 Val) is 71 000 M^{-1} s^{-1} and 29 000 with pentapeptides with a P_1 Ala (Bauer et al., 1976). With both enzymes, extension of the peptide chain of the substrate to encompass P_1' and P_2' residues results in over a 100-fold increase in k_{cat}/K_M. Our results indicate that there may be more bonds in elastin that are susceptible to PP elastase than are susceptible to HL elastase, not that HL elastase is a less reactive enzyme than PP elastase.

Cathepsin G. Cathepsin G is a major serine protease of leukocytes often referred to as "chymotrypsin-like." Its function is not yet known. In contrast to bovine chymotrypsin, however, the best tetrapeptide 4-nitroanilide substrate (MeO-Suc-Ala-Pro-Met-NA) for cathepsin G has a P_1 methionyl residue (Nakajima et al., 1978). With simple peptides, we cannot discern such a preference for Met at P_1 over Phe, although a P_1 Met is not significantly poorer. Tetrapeptides with a P_1 Val or Ala are not cleaved at all. Changing the P_5 Ac group to a MeO-Suc group had almost no effect on k_{cat}/K_M. Thus, with both cathepsin G and HL elastase, interaction (if any) of the enzyme with the P_5 group has no significant influence on peptide bond or 4-nitroanilide hydrolysis (Nakajima et al., 1978).

Cathepsin G seems to prefer a P_1' Phe slightly over either an Ala or Leu in the pentapeptide substrates. The pentapeptide with a P_1' Phe binds more tightly than that with either Ala or Leu, but the P_1' Ala peptide was cleaved more rapidly

than either. In the series of compounds with a P_1 Met residue, a P_1' Ser or Thr residue has a small effect (threefold). Ser is preferred in the octapeptide case while Thr is preferred with the hexapeptides. However, with both the octa- and hexapeptides, the P_1' Thr isomers were bound more tightly.

With cathepsin G, the length of the peptide chain has little effect on binding of the substrate. But the addition of a P_1' residue increases k_{cat} by a factor of 10–25. Further extension of the peptide chain thus far has not resulted in significant increases in k_{cat}/K_M values with cathepsin G. Also, it is clear that the maximum k_{cat}/K_M value yet observed with cathepsin G and peptide substrates is at least 50-fold lower than that which might be expected based on experience with other serine proteases such as HL elastase. Cathepsin G also exhibits low reactivity toward peptide chloromethyl ketone inhibitors (Powers et al., 1977) and peptide 4-nitroanilide substrates (Nakajima et al., 1978). Is cathepsin G simply a poor peptidase? Or is it an exceptionally specific enzyme with the proper substrate yet to be found? These are questions that can only be answered with further work.

α_1-Protease Inhibitor Reactive Site. α_1-Protease inhibitor (α_1-antitrypsin) inhibits a number of serine proteases such as HL and PP elastase, cathepsin G, bovine chymotrypsin, and trypsin. The sequence of the reactive site of α_1-PI has recently been shown to be -Ala-Ile-Pro-Met*Ser-Ile-Pro-Pro- where the asterisk indicates the bond split when α_1-PI·protease complexes are broken with a base or nucleophiles (Johnson & Travis, 1978).

We synthesized the octapeptide Ac-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro-NH_2 which has the P_4–P_4' sequence of the α_1-PI reactive site and an analogue with a P_1' Thr instead of Ser. These octapeptides were cleaved by HL elastase, PP elastase, and cathepsin G, three of the major enzymes which are inhibited by α_1-PI. The binding of the octapeptides to the various enzymes as measured by K_M values varied from 0.55 to 4.6 mM for the P_1' Ser peptide and from 1.9 to 3.6 mM for the P_1' Thr peptide. These values are orders of magnitude higher than the K_I values for α_1-PI·protease complexes and indicate that this particular sequence in itself is not sufficient to get tight binding to a serine protease. α_1-PI may effect tight binding by locking this sequence into a favorable conformation. Indeed, conformationally restricted peptides with related sequences bind much more tightly to HL elastase than the octapeptide (A. Yasutake, N. Nishino, and J. C. Powers, unpublished observations).

In addition to a simple association, interaction of serine proteases with α_1-PI involves some type of catalytic event. This process requires the active-site serine of the serine protease since anhydrotrypsin will not react with α_1-PI. Complexes of α_1-PI and serine proteases are stable to $NaDodSO_4$ gel electrophoresis, indicating a covalent linkage between α_1-PI and the protease. A tetrahedral adduct of the active-site serine of the protease to the peptide bond (P_1–P_1') at the reactive site of α_1-PI or an acyl enzyme has variously been postulated. A tetrahedral adduct has precedent in the structures of the trypsin–soybean trypsin inhibitor or pancreatic trypsin inhibitor complex (Sweet et al., 1974; Huber et al., 1974).

The k_{cat} values obtained with the octapeptides could be considered to be an indicator of the ease with which certain types of covalent bond formation (acylation) between α_1-PI and a serine protease takes place. At present, the type of bonding in α_1-PI·serine protease complexes is not known. In addition, the octapeptides are not conformationally restricted as the reactive site of α_1-PI must be. But with these uncertainties, it can be seen that the k_{cat} values for the octapeptides lie in a fairly narrow range from 1.5 to 16 s^{-1}. For each enzyme studied, the k_{cat} values for the P_1' Ser octapeptide were 3–4 times higher than those for the P_1' Thr octapeptide. In this regard, Odani & Ikenaka (1978) have recently chemically substituted a number of amino acids at the P_1' residue of the chymotrypsin reactive site of the Bowman–Birk inhibitor. The P_1' Ser inhibitor was found to have a 20-fold lower K_I value than the P_1' Thr inhibitor. Thus, it appears that relatively minor amino acid substitutions in the region of the α_1-PI reactive site will profoundly influence its reactivity.

A number of genetic variants of α_1-PI have been detected electrophoretically. Severe α_1-PI deficiency of the homozygous Z type is characteristic of early onset familial emphysema. This variant has been shown to have a Lys for Glu substitution somewhere in the molecule (Jeppsson et al., 1978). Current evidence seems to indicate that certain individuals with "normal" α_1-PI are more susceptible to emphysema than others. A rather simple substitution in the reactive-site region such as Thr for Ser substitution at P_1' could certainly account for a difference in the reactivity of α_1-PI and its ability to complex with free proteases before they have an opportunity to do any damage.

The octapeptide with the P_4–P_4' sequence of α_1-PI reacted with HL elastase, PP elastase, and cathepsin G, three of the major enzymes inhibited by α_1-PI. Pancreatic trypsin (TPCK treated), which is probably not the major target for α_1-PI, also reacted with the Met-containing hexapeptides, though at very low rates. The trypsin hydrolysis rates were ~1–2% of those for HL elastase and 5–10% of those for cathepsin G. The octapeptide was not tested with pancreatic trypsin due to a lack of material. Thus, although the amino acid sequence of the α_1-PI reactive site is not ideal for certain serine proteases such as trypsin, it is reactive toward a number of proteases with differing substrate specificity. In fact, evolutionary pressure would be expected to yield a molecular structure for the α_1-PI proteolysis site which would enable the inhibitor to react with many of the diverse serine proteases it might be exposed to.

Methionine Oxidation. Inactivation of α_1-PI occurs upon oxidation of the active-site Met residue (Johnson & Travis, 1979). Previously we had shown that oxidation of MeO-Suc-Ala-Ala-Pro-Met-NA to the sulfoxide completely destroyed its reactivity toward HL elastase and cathepsin G and substantially decreased its reactivity toward α-chymotrypsin (Nakajima et al., 1978). The data we obtained with simple peptides are summarized in Table IV. For each enzyme and peptide studied, the ratio of the kinetic constants obtained with the Met and the Met(O) derivative is reported.

In the vast majority of cases, oxidation of the Met residue to the sulfoxide decreased the binding of the peptide to the enzyme ($1/K_M$), k_{cat}, and k_{cat}/K_M. HL elastase, for example, binds 2.3 times better to the unoxidized octapeptide Ac-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro-NH_2 than to the corresponding Met(O) derivative and has a 170-fold higher k_{cat}. This indicates that oxidized α_1-PI should bind less tightly to HL elastase and should undergo bond formation with greater difficulty. In fact, the rate of association of HL elastase with oxidized α_1-PI is substantially decreased (Beatty et al., 1980), and the complex formed is unstable and is converted to a lower molecular weight form (Johnson & Travis, 1979).

Cigarette smoking decreases the elastase inhibitor capacity of α_1-PI in humans (Janoff et al., 1979; Gadek et al., 1979). This is probably due to the formation of oxidized α_1-PI either by reaction with some component in the smoke or by reaction with myeloperoxidase from leukocytes (Matheson et al., 1979).

Table IV: Effect of Methionine Oxidation on the Enzymatic Hydrolysis of Hexa- or Octapeptides Containing either a P_1' Ser or Thr

enzyme	P_1' residue (peptide length)[b]	ratio Met/Met(O)[a]		
		$1/K_M$	k_{cat}	k_{cat}/K_M
HL elastase	Ser (6)	2.1	2.4	5.4
	Ser (8)	2.3	170	390
	Thr (6)	2.5	1	2.5
	Thr (8)	1.7	46	79
PP elastase	Ser (6)	4.4	2.5	12
	Ser (8)	18	3.6	68
	Thr (6)	4.1	1.7	7.1
	Thr (8)	2.5	3.7	9.7
cathepsin G	Ser (6)	4.2	4	16
	Ser (8)	0.8	30	23
	Thr (6)	3.2	80	230
	Thr (8)	2.0	3.0	5.6

[a] Ratio of the various kinetic constants measured for the methionine-containing peptide relative to the identical peptide with a methionine sulfoxide. [b] 6 refers to Ac-Ala-Ala-Pro-Met-(Ser or Thr)-Ala-NH_2 and its oxidized derivative. 8 refers to Ac-Ala-Ile-Pro-Met-(Ser or Thr)-Ile-Pro-Pro-NH_2 and its oxidized derivative.

The protease unbalance thus created is thought to lead to the development of emphysema. The results we have obtained with simple peptides certainly lend additional support to this model for the genesis of emphysema.

Another interesting clinical observation is the fact that certain smokers seem more disposed to emphysema than others. Possibly this is linked to genetic variations in the reactive-site region of α_1-PI. Our studies show that the reduction of substrate effectiveness upon oxidation is greatest with HL elastase for the Ser-containing peptides. The effect is greatest in k_{cat}. A similar trend is observed with the other enzymes. Thus, one can conclude that genetic variations in the α_1-PI reactive site cannot only change its reactivity toward serine proteases but also could effect the degree to which oxidation is damaging to the inhibitor.

Supplementary Material Available

Experimental details for the synthesis of the new compounds reported (9 pages). Ordering information is given on any current masthead page.

References

Bauer, C. A., Thompson, R. C., & Blout, E. R. (1976) *Biochemistry 15*, 1296.

Beatty, K., Bieth, J., & Travis, J. (1980) *J. Biol. Chem. 255*, 3931.

Bieth, J., & Wermuth, C. G. (1973) *Biochem. Biophys. Res. Commun. 53*, 383.

Blondin, J., Janoff, A., & Powers, J. C. (1978) in *Neutral Proteases of Human Polymorphonuclear Leukocytes* (Havemann, K., & Janoff, A., Eds.) pp 39–55, Urban & Schwarzenberg, Baltimore and Munich.

Gadek, J., Felles, G. A., & Crystal, R. G. (1979) *Science (Washington, D.C.) 206*, 1315.

Hance, A. J., & Crystal, R. G. (1975) *Am. Rev. Respir. Dis. 112*, 656.

Huber, R., Kukla, D., Steigenmann, W., Deisenhofer, J., & Jones, A. (1974) *Bayer-Symp. 5*, 497–512.

Janoff, A., Carp, H., Lee, D. K., & Drew, R. T. (1979) *Science (Washington, D.C.) 206*, 1313.

Jeppsson, J. O., Laurell, C. B., & Fagerhol, M. (1978) *Eur. J. Biochem. 83*, 143.

Johnson, D., & Travis, J. (1978) *J. Biol. Chem. 253*, 7142.

Johnson, D., & Travis, J. (1979) *J. Biol. Chem. 254*, 4022.

Matheson, N. R., Wong, P. S., & Travis, J. (1979) *Biochem. Biophys. Res. Commun. 88*, 402.

Mittman, C., Ed. (1972) *Pulmonary Emphysema and Proteolysis*, pp 1–537, Academic Press, New York.

Nakajima, K., Powers, J. C., Ashe, B. M., & Zimmerman, M. (1978) *J. Biol. Chem. 254*, 4027.

Odani, S., & Ikenaka, T. (1978) *J. Biochem. (Tokyo) 84*, 1.

Powers, J. C., & Tuhy, P. M. (1973) *Biochemistry 12*, 4767.

Powers, J. C., & Gupton, B. F. (1977) *Methods Enzymol. 46*, 208.

Powers, J. C., Gupton, B. V., Harley, A. D., Nishino, N., & Whitley, R. J. (1977) *Biochim. Biophys. Acta 485*, 165.

Powers, J. C., Gupton, B. F., Lively, M. O., Nishino, N., & Whitley, R. J. (1978) in *Neutral Proteases of Human Polymorphonuclear Leukocytes* (Havemann, K., & Janoff, A., Eds) pp 221–233, Urban & Schwarzenberg, Baltimore and Munich.

Rosen, H. (1957) *Arch. Biochem. Biophys. 67*, 10.

Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157.

Segal, D. M. (1972) *Biochemistry 11*, 349.

Senior, R. M., Tegner, H., Kuhn, C., Ohlsson, K., Starcher, B. C., & Pierce, J. A. (1977) *Am. Rev. Respir. Dis. 116*, 469.

Sweet, R. M., Wright, H. T., Janin, J., Chothia, C. H., & Blow, D. M. (1974) *Biochemistry 13*, 4212.

Thompson, R. C., & Blout, E. R. (1973a) *Biochemistry 12* 44.

Thompson, R. C., & Blout, E. R. (1973b) *Biochemistry 12*, 51.

Thompson, R. C., & Blout, E. R. (1973c) *Biochemistry 12*, 57.

Thomson, A., & Denniss, I. S. (1973) *Eur. J. Biochem. 38*, 1.

Tuhy, P. M., & Powers, J. C. (1975) *FEBS Lett. 50*, 359.

Turino, G. M, Rodriquez, J. R., Greenbaum, L. M., & Mandl, I. (1974) *Am. J. Med. 57*, 493.

Visser, L., & Blout, E. R. (1972) *Biochim. Biophys. Acta 268*, 257.

Zimmerman, M., & Ashe, B. M. (1977) *Biochim. Biophys. Acta 480*, 241.