**Pseudomonas aeruginosa Elastase**

DEVELOPMENT OF A NEW SUBSTRATE, INHIBITORS, AND AN AFFINITY LIGAND*

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The elastase produced by *Pseudomonas aeruginosa* is probably responsible for the tissue destruction observed during pulmonary and corneal infections by this pathogen. We have synthesized a new substrate, Abz-Ala-Gly-Leu-Ala-Nba, for *P. aeruginosa* elastase. Cleavage of the peptide by elastase at pH 7.2 results in 6- to 7-fold increase in fluorescence (λem, 340 nm; λex, 415 nm). A sensitive rate assay utilizing this substrate was developed and used to study inhibitors. Elastase was irreversibly inhibited by the tripeptide analog ClCH₂CO-HOLeu-OCH₃, a thermolysin inhibitor, but the hydroxamic acid (HONHCOCH(CH₃)CO-Ala-Gly-NH₂) lacking the benzyl side chain was a 6000-fold insensitive rate assay utilizing this substrate was developed and used to study inhibitors. Elastase has an extended substrate recognition site. Some of the inhibitors may find use in the clinical treatment of *P. aeruginosa* infections. The abbreviations used are: Abz, 2-aminobenzoyl; Nba, N-hydroxypeptide (CHO-HOLeu-Ala-Gly-NH₂). The substrate specificity of the enzyme is that of Schechter and Berger (1967). The elastase is a zinc metalloendoprotease (0.9 Zn/mol) and is inhibited by EDTA, Zn²⁺, and o-phenanthroline (Morihara et al., 1965; Morihara and Tsuzuki, 1978). The substrate specificity of the *P. aeruginosa* elastase resembles that of thermolysin with peptides that contain a P1 leucyl or phenylalanyl residue being split most rapidly (Morihara and Tsuzuki, 1966 and 1971; Morihara and Ebata, 1967; Morihara et al., 1968). Phosphoramidon, a potent inhibitor of thermolysin which is isolated from culture filtrates of actinomycetes (Komiyama et al., 1975), is also an excellent inhibitor of *P. aeruginosa* elastase (Morihara and Tsuzuki, 1978).

We have recently developed a number of reversible and irreversible hydroxamic acid inhibitors (Nishino and Powers, 1978; Rasnick and Powers, 1978) of thermolysin and used one of the inhibitors as a ligand in the affinity chromatography of thermolysin (Nishino and Powers, 1979). We are currently extending these inhibitors to other metalloproteases. In this paper, we report studies with a number of synthetic peptide derivatives which we have shown to inhibit the *P. aeruginosa* elastase. In addition, we have developed a new assay substrate for the enzyme, synthesized an irreversible inhibitor, and developed a method for affinity purification of the enzyme.

**MATERIALS AND METHODS**

* Materials and Methods used for the development of the enzyme were identical to those described previously.

1. The nomenclature used for the individual amino acid residues (P₁, P₂, P₃, etc.) of a substrate or inhibitor and the substrates (S₁, S₂, S₃, etc.) of the enzyme is that of Schechter and Berger (1967).

2. Portions of this paper (including "Materials and Methods" and Figs. 1 to 3) are presented in miniprint. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, Md. 20014. Request Document No. 79M-2026, cite authors, and include a check or money order for $1.00 per set of photocopies.

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Elastase solution was prepared as described above from *P. aeruginosa* strain NCTC 7086. Portions of this paper (including "Materials and Methods" and Figs. 1 to 3) are presented in miniprint. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, Md. 20014. Request Document No. 79M-2026, cite authors, and include a check or money order for $1.00 per set of photocopies.
RESULTS

One of our first goals in the study of *P. aeruginosa* elastase was the development of a sensitive and convenient assay for the enzyme. Prior to our work, the most useful assay was the Z-Gly-Leu-NH$_2$ assay developed by Morihara and Ebata (1967). This involves following the rate of cleavage of the Gly-Leu bond by analyzing for the H-Leu-NH$_2$ produced using the ninhydrin method. This assay is time-consuming and not readily adaptable to continuous monitoring of the hydrolysis reaction.

Since the substrate specificity of *P. aeruginosa* elastase is quite similar to that of thermolysin, we decided to investigate the hydrolysis of furylacryloyl-Gly-Leu-NH$_2$. The hydrolysis of this substrate by thermolysin can be followed spectrophotometrically (Feder, 1968), but its $K_m$ value is quite high ($K_m = 30$ mM, $k_{cat}/K_m = 1.6 \times 10^4$ M$^{-1}$ s$^{-1}$). At pH 7.2, in a Tris buffer, furylacryloyl-Gly-Leu-NH$_2$ was hydrolyzed by *P. aeruginosa* elastase ($k_{cat}/K_m = 4.9 \times 10^3$ M$^{-1}$ s$^{-1}$). However, it was quite a poor substrate and, due to its low solubility, was unsuitable for the study of inhibitors with low $K_I$ values.

Fluorogenic Substrate—One fairly new method for the assay of proteolytic enzymes involves the use of peptides containing both a fluorescent and fluorescence-quenching group. Hydrolysis of the peptide with a resulting separation of the fluorescent and quenching groups results in an increase in the fluorescence which can be used to follow the hydrolysis rate. This technique has been used in assays for carboxypeptidase (Latt et al., 1972), trypsin (Carmel et al., 1973), leucine aminopeptidase (Carmel et al., 1977), and the angiotensin-converting enzyme (Persson and Wilson, 1977). Abz-Ala-Gly-Leu-Ala-Nba$_3$ was synthesized as a possible substrate for the *P. aeruginosa* elastase since it has the same sequence as Z-Ala-Gly-Leu-Ala-OH which is one of the most susceptible substrates of elastase (Morihara and Tsuzuki, 1969). The abbreviations used are: Abz, 2-aminobenzoyl; Nba, NHCH$_2$C$_6$H$_4$NO$_2$ (4-nitrobenzylamide); HOLeu, N-hydroxyleucine; unless otherwise noted all amino acids except glycine have the L-configuration; SDS, sodium dodecyl sulfate.

![Fluorescence of Abz-Ala-Gly-Leu-Ala-Nba$_3$-H$_2$O and the hydrolysate products (Abz-Ala-Gly-Leu-Ala-Nba$_3$, HOAla-OH, and 4-nitrobenzylamide) after digestion with *Pseudomonas aeruginosa* elastase (closed circles).](image1.png)

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Reversible Inhibitors-The fluorogenic substrate Abz-Ala-Gly-Leu-Ala-Nba was very susceptible to hydrolysis by P. aeruginosa elastase. The enzyme concentration used in this study was 1.7 nM and, although we did not attempt to determine the limit of sensitivity, it was obviously a highly sensitive assay for the enzyme.

Cleavage at any one of the peptide bonds of Abz-Ala-Gly-Leu-Ala-Nba would result in an increase in fluorescence due to separation of the fluorogenic and the quenching group. Based on the work of Morihara and Tsuzuki (1971) with a variety of peptides, the Gly-Leu bond would be the bond most susceptible to cleavage. The fluorogenic substrate Abz-Ala-Gly-Leu-Ala-Nba was linear up to 0.03 mM. However, at concentrations from 0.0125 to 0.2 mM, the initial rate of hydrolysis of the substrate was linear up to 15% hydrolysis.

Reversible Inhibitors—The $K_i$ values for a number of reversible inhibitors of P. aeruginosa elastase are given in Table 1 along with the corresponding values for thermolysin. The inhibition mode was shown to be competitive. Many of the compounds are extremely potent inhibitors for both enzymes, with the hydroxamic acid HONHCOCH(CH$_2$CH$_3$)CO-Ala-Gly-NH$_2$, having the lowest $K_i$ value (44 nM) with elastase. The two hydroxamic acids with a phenylalanyl or leucyl side chain were almost equally potent. However, the hydroxamic acid with no side chain, HONHCOCH(CH$_3$)CO-Ala-Gly-NH$_2$, was over 6000-fold poorer. The N-hydroxy peptide CHO-HOLeu-Ala-Gly-NH$_2$, which is a structural isomer of the hydroxamic acid, HONHCOCH(CH$_2$CH$_3$)CO-Ala-Gly-NH$_2$, was a 130-fold poorer inhibitor of elastase. The thiol compound was also almost as potent as the best hydroxamic acid.

Phosphoramidon has previously been shown to be both an inhibitor of P. aeruginosa elastase (Morihara and Tsuzuki, 1978) and thermolysin (Komiyama et al., 1975). The $K_i$, which we obtained for elastase (57 nM) using Abz-Ala-Gly-Leu-Ala-Nba as a substrate at pH 7.2 at 25°C, compares quite favorably with that reported (40 nM) using Z-Gly-Leu-NH$_2$ at pH 7.0 and 40°C. One distinct difference in the inhibition of elastase and thermolysin with Phosphoramidon was observed. With thermolysin, preincubation with the inhibitor for 10 to 15 min is required to obtain maximum inhibition due to a slow formation of the E-I complex (Kam et al., 1979). With P. aeruginosa elastase, maximum inhibition is reached immediately.

Affinity Chromatography—The synthesis of the affinity resin HONHCOCH(CH$_2$CH$_3$)CO-Ala-Gly-NH$_2$(CH$_3$)$_2$-agaroose, with one of the best hydroxamic acid inhibitors as a ligand, has recently been accomplished (Nishino and Powers, 1979). This resin would bind thermolysin, but would not bind chymotrypsin A, or carboxypeptidase A. As can be seen in Fig. 2, P. aeruginosa can also be successfully purified using this resin. About 80% of the 280 nm-absorbing material was washed through the column with a pH 7.2 buffer. This eluate contained less than 1% of the original enzyme activity. A pH 8.6 buffer eluted a small amount of activity (less than 5%). The majority of the elastase activity was eluted with a 0.1 M Tris solution containing 0.1 M CaCl$_2$ at pH 10.0. The recovery of activity in the two 2-ml fractions was 92%, although this corresponded to only 21% of the original UV absorbance. The pH of the two combined fractions was 9.4. A pH 9.0 buffer which affords sharp emergence of thermolysin from the column (Nishino and Powers, 1979) eluted elastase in a broad peak over 10 fractions. This could be the result of tighter binding of elastase to the column, since the $K_i$ value of HONHCOCH(CH$_3$)CO-Ala-Gly-NH$_2$ with elastase (44 nM) is lower than that with thermolysin (300 nM). It is reported that elastase is stable in the pH range 6 to 10 (Morihara et al., 1965). This stability was confirmed by the rechromatography. The pH of the eluate was neutralized with acetic acid and reapplied to the column. After elution with the same system, 98% of the activity was recovered. No peak was eluted with the pH 8.6 buffer upon rechromatography. The purity of the chromatographed sample was examined by SDS-gel electrophoresis. A sample was dialyzed in 0.1 M Tris-HCl, 2.5 mM CaCl$_2$, containing 0.4 $\mu$M HONHCOCH(CH$_3$)CO-Ala-Gly-NH$_2$ at 4°C overnight to reduce the Ca$^{2+}$ concentration. A single band with a $M_r$ ~ 21,000 was observed. Dialysis without inhibitor gave minor bands (three), with smaller molecular weight, probably produced by autolysis.

Irreversible Inhibition—Thermolysin is irreversibly inhibited by CICH$_2$CHO-HOLeu-OCH$_3$ (Rasnick and Powers, 1979). Incubation of P. aeruginosa elastase with CICH$_2$CHO-HOLeu-OCH$_3$ (50 mM) showed a small decrease in activity due to reversible binding of the inhibitor to elastase. However, elastase retained over 80% of its activity after 3 days of incubation at 25°C with a 50 mM concentration of CICH$_2$CHO-HOLeu-OCH$_3$ compared to a control which lacked inhibitor. The half-life for the inhibition of thermolysin under these conditions would have been a few minutes.

We then decided to test whether extension of the peptide chain would have an effect on the inhibition. Peptide chain extension has been shown to profoundly influence the rate of reaction of peptide chloromethyl ketone inhibitors with porcine pancreatic elastase (Powers and Tuhy, 1973). The synthesis of CICH$_2$CHO-HOLeu-Ala-Gly-NH$_2$ was accomplished.
and incubation of this compound with *P. aeruginosa* elastase resulted in a progressive loss of enzyme activity.

The kinetics of inhibition of certain enzymes by irreversible inhibitors reveals the presence of a reversible complex between enzyme and inhibitor preceding covalent bond formation. The reaction can be described by Equation 1 where $E-I$ represents a noncovalently bound complex of enzyme with inhibitor and $E-I$ is the final product with the inhibitor irreversibly bound to the enzyme via a covalent linkage (Kitz and Wilson, 1962).

$$E + I \xrightleftharpoons{K_I} E-I \underset{k_2}{\rightarrow} E-I$$

The observed pseudo-first-order rate constant for inhibition should be a function of inhibitor concentration and can be expressed in a convenient reciprocal form (Equation 2) where $K_I$ is the dissociation constant for the enzyme inhibitor complex.

$$\frac{1}{k_{obs}} = \frac{1}{k_I} + \frac{1}{k_s}$$

However, when the rate of inactivation of *P. aeruginosa* elastase by CIC\textsubscript{3}H\textsubscript{2}CO-HOLeu-Ala-Gly-NH\textsubscript{2} was measured at several higher inhibitor concentrations ($[I] = 10$ to $40$ mM) in a pH 7.2 buffer at 25°C, no distinguishable difference in rate (half-life ~ 90 min) was observed throughout the range of inhibitor concentration used. The observed rate constant was $1.2 \times 10^{-4}$ s\textsuperscript{-1} ($k_{obs}$). By utilizing short reaction times and a Dixon plot, a $K_I$ value for CIC\textsubscript{3}H\textsubscript{2}CO-HOLeu-Ala-Gly-NH\textsubscript{2} of 1.3 mM was measured. Therefore, enzyme was saturated at the higher inhibitor concentration used for the inactivation kinetic studies. In the case $[I] \gg K_I$, $k_s$ is equal to $k_{obs}$ which makes the $k_s/K_I = 0.992$ M\textsuperscript{-1} s\textsuperscript{-1}.

Thermolysin was inactivated by CICH\textsubscript{3}CO-HOLeu-Ala-Gly-NH\textsubscript{2} (20 mM) much faster than by CIC\textsubscript{3}H\textsubscript{2}CO-HOLeu-OMe (Rasnack and Powers, 1978) giving $k_{obs} = 40$ m\textsuperscript{-1} s\textsuperscript{-1}. Assuming a $K_I$ value for the tripeptide of ~2 mM (the $K_I$ of Ac-DL-HOLeu-Ala-Gly-NH\textsubscript{2} is 3.4 mM, Nishino and Powers, 1979), $k_{obs}/[I] = k_s (K_I + [I]) = k_s/K_I$ (when $K_I \gg [I]$) = 40 m\textsuperscript{-1} s\textsuperscript{-1}. The $k_s/K_I$ for CICH\textsubscript{3}CO-HOLeu-OMe is 1.0 m\textsuperscript{-1} s\textsuperscript{-1} (Rasnack and Powers, 1978).

A *P. aeruginosa* competitive inhibitor was also demonstrated to protect the active site against an irreversible inhibition. Four-hundred micromolar concentration of HO-\textsubscript{N}NHCO\textsubscript{CH}(CH\textsubscript{3})\textsubscript{2}CO-HOLeu-Ala-Gly-NH\textsubscript{2} ($7 \times K_I$) doubled the half-life for the inhibition reaction and gave $k_{obs} = 6.2 \times 10^{-5}$ s\textsuperscript{-1}. This agrees quite well with the $0.992$ m\textsuperscript{-1} s\textsuperscript{-1} calculated from $k_{obs} = k_I/[I]/(K_I + [I]) + [I]$, where $I$ is the concentration of the competitive inhibitor and $K_I$ is its dissociation constant.

**DISCUSSION**

*P. aeruginosa* elastase is a zinc metalloprotease secreted by many pathogenic strains of *P. aeruginosa*. Hemorrhages of the lungs and corneal ulcers have been associated with infections by this organism and it is probable that elastase is causing much of the tissue destruction. The goal of our research with this enzyme was to develop good inhibitors for this elastase and to investigate its relationship to other zinc metalloproteases such as thermolysin.

Peptide hydroxamic acids are excellent reversible inhibitors of thermolysin (Nishino and Powers, 1978) and we have now shown them to be potent inhibitors of *P. aeruginosa* elastase. In fact, both HONHCO\textsubscript{CH}(CH\textsubscript{3})\textsubscript{2}CO-Ala-Gly-NH\textsubscript{2} and HONHCO\textsubscript{CH}(CH\textsubscript{3})\textsubscript{2}CO-Ala-Gly-NH\textsubscript{2} bind to *P. aeruginosa* elastase 10-fold more tightly than they do to thermolysin. The hydroxamic acid HONHCO\textsubscript{CH}(CH\textsubscript{3})\textsubscript{2}CO-Ala-Gly-NH\textsubscript{2} with no side chain is an over-6000-fold poorer inhibitor. This is consistent with the substrate specificity of *P. aeruginosa* elastase which splits substrates with a P\textsubscript{1} phenylalanine or leucine residue quite effectively, while those with a P\textsubscript{1} glycine are not split (Morihara and Tsuzuki, 1971 and 1975). The side chain specificity exhibited by the hydroxamic acids indicate that they are reacting with the S\textsubscript{1} substrate of elastase. The alanine residue at P\textsubscript{2} is also a favorable residue in substrates (Morihara and Tsuzuki, 1971 and 1975). The proposed mode of binding of a hydroxamic acid to elastase is shown in Fig. 4. The P\textsubscript{1} side chain is interacting with the S\textsubscript{1} residue, and the hydroxamic acid functional group is acting as a bidentate ligand for the active site zinc atom. We have proposed a similar model to account for the inhibition of thermolysin by peptide hydroxamic acids.

The N-hydroxy peptide CH\textsubscript{3}CO-HOLeu-Ala-Gly-NH\textsubscript{2} is a structural isomer of the hydroxamic acid HONHCO\textsubscript{CH}(CH\textsubscript{3})\textsubscript{2}CO-Ala-Gly-NH\textsubscript{2}. However, it is a 130-fold poorer inhibitor. This difference cannot be due simply to differences in the coordinating ability of the two functional groups since a much smaller difference (6-fold) is observed with thermolysin. The hydroxamic acid functional group is capable of interacting more strongly with the hydroxamic acid functional group than the N-hydroxy-peptide and more effectively than thermolysin.

Cushman et al. (1977) have recently shown that mercaptoalkanoyl amino acids are potent inhibitors of the angiotensin-converting enzyme. In fact, the best inhibitor HSCH\textsubscript{2}CH(CH\textsubscript{3})\textsubscript{2}CO-Pro-OH had a $K_I$ of 1.7 nM. We have now extended this class of inhibitor to the *P. aeruginosa* elastase by showing that HSCH\textsubscript{2}CH(CH\textsubscript{3})\textsubscript{2}CO-Ala-Gly-NH\textsubscript{2} is a potent inhibitor (64 nM). This is undoubtedly binding to elastase in the same fashion as the hydroxamic acids except with the thiol group acting as the zinc ligand (Fig. 4). Again, elastase is capable of stronger interaction with the inhibitor than thermolysin.

*P. aeruginosa* is inhibited by Phosphoramidon (rhamnose-P-Leu-Trp-OH), an inhibitor of thermolysin which contains a phosphoramidate functional group (Morihara and Tsuzuki, 1978; Komiyama et al., 1975). An x-ray crystallographic study of the thermolysin-Phosphoramidon complex has shown that the leucyl residue of the inhibitors binds to the S\textsubscript{1} subsite of thermolysin and one of the phosphoramidate oxygens is acting as a ligand for the active site zinc atom of the enzyme (Weaver et al., 1977). In fact, the $K_I$ values obtained with Phosphoramidon and the two enzymes are quite similar. Therefore, there must be considerable similarity in both catalytic sites and substrate binding regions of thermolysin and *P. aeruginosa* elastase.
There is, however, one distinct difference between the two enzymes. *P. aeruginosa* elastase is inhibited immediately by Phosphoramidon, while preincubation of thermolysin with Phosphoramidon for 10 to 15 min is required for complete inhibition.

It is now clear that a number of metalloproteases can be inhibited by peptides containing the same functional groups. Peptide hydroxamic acids and thiols have been shown to inhibit thermolysin, *P. aeruginosa* elastase, and the angiotensin-converting enzyme (Nishino and Powers, 1978; Cushman et al., 1977). Phosphoramidates will inhibit *P. aeruginosa* elastase, thermolysin, and carboxypeptidase A (Kam et al., 1979). Although these enzymes differ in their substrate specificity, it is clear that their catalytic sites are all closely related as evidenced by their inhibition by the same types of inhibitors. Some of the enzymes bind the inhibitors much more tightly. For example, *P. aeruginosa* elastase binds the thiol and hydroxamic acids 10-fold more tightly than thermolysin. This points to subtle differences in their active site geometries.

Thromolysin is irreversibly inhibited by CICH₂CO-DL-HOLeu-OCH₃ due to alkylation of the active site glutamic acid residue (Glu-143) (Rasnitz and Powers, 1978). *P. aeruginosa* elastase, on the other hand, was completely inert to this reagent. However, it was inhibited by CICH₂CO-HOLeu-Ala-Gly-NH₂. This shows that elastase, unlike thromolysin, requires an extended peptide structure for inhibition. This behavior is quite reminiscent of porcine pancreatic elastase which is inhibited by tri- and tetrapeptide chloromethyl ketone inhibitors, but slowly, or not at all, by dipeptide or amino acid chloromethyl ketones (Powers and Tuhy, 1973; Powers, 1977).

In conclusion, we have developed an excellent substrate and affinity purification method for *P. aeruginosa* elastase which should facilitate future studies with the enzyme. Several potent reversible and irreversible inhibitors have been described. Such inhibitors may be of value in the future for the clinical treatment of *P. aeruginosa* infections.

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**REFERENCES**


Powers, J. C., and Tuhy, P. M. (1973) *Biochemistry* 12, 4767-4774

