SPECIFIC INHIBITION OF HUMAN GRANULOCYTE ELASTASE BY CIS-UNSATURATED FATTY ACIDS AND ACTIVATION BY THE CORRESPONDING ALCOHOLS

Bonnie M. Ashe and Morris Zimmerman
Merck Institute for Therapeutic Research
Rahway, N.J. 07065

Received January 13,1977

Summary

Human granulocyte elastase is markedly inhibited by cis-unsaturated fatty acids, whereas trypsin, chymotrypsin, pancreatic elastase, and the granulocyte chymotrypsin-like enzyme are totally unaffected. The most potent of the acids tested is oleic acid (K_i = $9x10^{-6}$M). The inhibition is non-competitive, affecting K_{cat} but not K_m with a tetrapeptide nitroanilide substrate. On the other hand, the corresponding alcohols and nitriles markedly stimulate the activity of granulocyte elastase on synthetic substrates but not on elastin, in contrast to the inhibitors which affect activity on both types of substrate. These data further define the differences between the granulocyte and pancreatic elastases and suggest that part of the difference may reside in the presence of an unusual hydrophobic binding site on the enzyme affecting its activity.

Introduction

Human granulocyte elastase has been implicated in a number of inflammatory diseases, both in the production of mediators of inflammation (1) and in the destruction of connective tissue (2-4). As part of a study of the role of the proteases in chronic inflammatory disease, and in an attempt to inhibit these enzymes selectively, we observed that certain unsaturated fatty acids inhibit human granulocyte elastase without affecting the other related serine proteases. In addition, corresponding unsaturated long-chain alcohols were observed to markedly stimulate the activity of granulocyte elastase with synthetic substrates.

Materials and Methods

Oleic and linoleic acids were obtained from Fisher Scientific Co., elaidic acid from Aldrich Chem. Co., palmitic and stearic acids from Supelco, Inc., linolenic acid from Applied Sciences, Inc., and arachidonic acid from P. L. Biochemicals. The remaining fatty acids and all of the alcohols were obtained from Nu Chek Prep Labs.

Purified human granulocyte elastase was prepared essentially according to the procedure of Taylor and Crawford (5). Purified human granulocyte chymotrypsin-like enzyme was prepared by a modification of the aforementioned procedure, involving chromatography on CM-sephadex C-50 according to Schmidt and Havemann (6). Hog pancreatic elastase, chromato-

ISSN 0006-291X

graphically purified, was obtained from Miles Laboratories. Bovine α-chymotrypsin and trypsin, both 3X crystallized, were obtained from Worthington Biochemical Corporation.

The elastase substrate, N-t-Boc-Ala-Ala-Pro-Ala-p-nitroanilide, and the chymotrypsin substrate, N-Acetyl-Ala-Ala-Pro-Phe-p-nitroanilide, were prepared in this laboratory (7). α-N-Benzoyl-D,L-arginine-p-nitroanilide HCl (BAPA) and t-Boc-L-Tyrosine-p-Nitrophenyl ester were purchased from Bachem, Inc. PIPES for buffers was purchased from Calbiochem. Elastin was obtained from Worthington Biochemical Corporation.

Both elastases were assayed with 0.2 mM N-t-Boc-Ala-Ala-Pro-Ala-p-nitroanilide in 0.05M K_2HPO_4/KH_2PO_4 buffer, pH 7.5, containing 10% dimethylsulfoxide (Me_2SO), at 25°C by measuring the production of nitroaniline at 410 nm using a Gilford spectrophotometer. Enzyme concentrations of 0.1 μM were used. Chymotrypsin and trypsin were assayed similarly using 0.2 mM N-Acetyl-Ala-Ala-Pro-Phe-p-nitroanilide and 1 mM BAPA respectively. Human granulocyte chymotrypsin-like enzyme was assayed with 0.2 mM t-Boc-L-Tyrosine-p-nitrophenyl ester in 0.05M PIPES[1] buffer, pH 6.5, containing 10% Me_2SO, at 25°C by measuring the production of p-nitrophenol at 347.5 nm. Elastase was also assayed spectrofluorometrically with elastin as substrate according to Quinn and Blout (8). A two-hour incubation time was used for these assays, and fluorescence was determined on a Perkin-Elmer 204-A fluorescence spectrophotometer.

Inhibitors, previously dissolved in Me_2SO, were added to the substrate solution at specified concentrations prior to the addition of the enzyme. Residual enzyme activity was compared to that of a control in which Me_2SO without inhibitor had been added. Activation studies were done similarly, enhanced enzyme activity being compared to that of a control in which Me_2SO without activator had been added to the substrate. Controls without enzyme were routinely run at the same time.

Results and Discussion

Shown in Table I are the effects of various long chain acids on the hydrolysis of t-Boc-Ala-Ala-Pro-Ala-p-nitroanilide by human granulocyte elastase. Those acids which showed no inhibition at a concentration of 0.2 mM are reported as inactive. At higher concentrations most of the acids precipitate from the substrate solution.

It is readily apparent that only unsaturated acids are inhibitors. Stearic and palmitic acids do not inhibit at the limits of solubility, nor does SDS, ruling out a possible detergent effect. There are fairly stringent conformational requirements since only cis acids, such as oleic, show activity while their trans isomers, such as elaidic acid, are inactive. Increasing the degree of unsaturation decreases the inhibitory capacity of the acid

[1] piperazine-N,N'-bis(2-ethanesulfonic acid), monosodium salt, monohydrate.

Table I

Inhibition of Human Granulocyte Elastase by Long Chain Acids

Long Chain Acid		Trivial Name	ID_{50}[a] (μg/ml)
14:1	cis-9	myristoleic	NA[b]
16:0		palmitic	NA
16:1	cis-9	palmitoleic	35
16:1	trans-9	palmitelaidic	NA
17:1	cis-10		13
18:0		stearic	NA
18:1	cis-6	petroselinic	10
18:1	trans-6	petroselaidic	NA
18:1	cis-9	oleic	2
18:1	trans-9	elaidic	NA
18:1	cis-11	vaccenic	10
18:1	trans-11	trans-vaccenic	NA
18:2	cis-9, cis-12	linoleic	8
18:2	trans-9, trans-12	linelaidic	NA
18:3	cis-9, cis-12, cis-15	linolenic	30
18:3	cis-6, cis-9, cis-12	gamma linolenic	NA
20:1	cis-11		4
20:4	cis-5, cis-8, cis-11, cis-14	arachidonic	15
20:3		8, 11, 14-eicosatriynoic	NA
22:1	cis-13	erucic	5
22:1	trans-13	brassidic	NA
24:1	cis-15	nervonic	10
Sodium Dodecyl Sulfate			NA

[a] 50% Inhibitory dose - the concentration which inhibits 50% of the enzyme activity under assay conditions described in text, using the tetrapeptide nitroanilide substrate.

[b] Not active

(oleic > linoleic >> linolenic). Eicosatriynoic acid is inactive. There also seems to be a minimum chain length requirement for inhibition; myristoleic acid does not inhibit at all, palmitoleic is slightly inhibitory whereas several C_{18} acids are good inhibitors.

The K_i's for four of the best inhibitors are given in Table II. The inhibition is non-competitive, as determined by Lineweaver-Burk double reciprocal plots, affecting K_{cat} but not K_m. There is no pH effect on inhibition over the range in which the enzyme is most active, pH 7.0-9.0.

Table II

Inhibitor Constants

Acid	K_i
Petroselinic	1.6×10^{-5} M
Oleic	9×10^{-6} M
Vaccenic	1.5×10^{-5} M
Linoleic	2.4×10^{-5} M

These unsaturated long chain acids are very specific inhibitors of granulocyte elastase. They have no effect on pancreatic elastase, trypsin, chymotrypsin, or the human granulocyte chymotrypsin-like enzyme, cathepsin G (9).

Whereas the tetrapeptide nitroanilide substrate has been used for most of our studies, we also determined the effect of these fatty acid inhibitors on granulocyte elastase when other substrates were employed. Oleic acid inhibits both the hydrolysis of t-Boc-Ala-p-nitrophenyl ester and the hydrolysis of elastin, using the spectrofluorometric assay of Quinn and Blout (8). The action of 40 μg of granulocyte elastase against elastin is inhibited 70% by 50 μg of oleic acid and inhibited 90% by 100 μg of oleic acid.

The presence of a binding site for unsaturated fatty acids on a membrane enzyme has been reported. Superactivation of isoproterenol-stimulated adenylate cyclase by unsaturated acids has virtually the same chain length and cis-trans specificity reported here (10).

In order to determine whether the acid moiety itself is necessary for inhibition, we tested several related long chain compounds, varying the terminal functional group. As shown in Table III, none of the long chain non-acids are inhibitors, but the alcohols and oleyl cyanide markedly stimulate granulocyte elastase activity with the nitroanilide substrate. Again only

Table III

Stimulation of Human Granulocyte Elastase by Long Chain Alcohols and Related Compounds

Compound	V_a/V_o
palmitoleyl alcohol	8
petroselinyl alcohol	12
oleyl alcohol	9
elaidyl alcohol	1
11-cis-eicosenol	9
erucyl alcohol	9
oleyl cyanide	4
oleyl amine	1
oleic monoethanol amide	1

V_a/V_o = Ratio of rate of nitroaniline release in presence of 0.1mM of compound to rate in absence of compound.

cis-unsaturated alcohols show activity; the trans isomers have no significant effect. These alcohols affect only K_{cat}, not K_m, and the degree of stimulation is directly proportional to alcohol concentration up to the limits of solubility.

Stimulation of activity is also specific for granulocyte elastase; pancreatic elastase, trypsin, chymotrypsin, and cathepsin G are totally unaffected by these alcohols. However, in contrast to the inhibitors which are active independent of the substrate used, the fatty alcohols do not stimulate the activity of granulocyte elastase against elastin. They do stimulate the hydrolysis of t-Boc-Ala-p-nitrophenyl ester, but to a much lesser degree than they stimulate nitroanilide hydrolysis. These findings are similar to the substrate-specific stimulation of chymotrypsin by azobenzenes, reported by Erlanger *et al.* (11).

Human granulocyte elastase is similar to pancreatic elastase in that it possesses an extended active site, is able to hydrolyze elastin and synthetic substrates such as t-Boc-Ala-p-nitrophenyl ester, and is inhibited by DFP, PMSF[1],

[1]Phenylmethylsulfonyl Fluoride

and α_1-antitrypsin. However it differs from pancreatic elastase in its substrate specificity, having a broader specificity for longer chain aliphatic amino acids at the bond cleaved (7). It is also inhibited more slowly by derivatives based on alanine analogs, such as elastatinal and chloromethyl ketones. The data presented in this paper, in addition to defining another difference between the granulocyte enzyme and its pancreatic counterpart, suggest that part of the difference may reside in the presence of an unusual hydrophobic binding site affecting, perhaps, the conformation of the enzyme when the appropriate compound is bound to that site. Direct examination of this possibility using circular dichroism could not be attempted due to the insolubility of the compounds at the levels necessary to affect the high enzyme concentrations required for such studies.

References

1. Janoff, A. (1972) Ann. Rev. Med. 23, 177-190.
2. Janoff, A., Feinstein, G., Malemud, C., and Elias, J. M. (1976) J. Clin. Invest. 57, 615-624.
3. Keiser, H., Greenwald, R. A., Feinstein, G., and Janoff, A. (1976) J. Clin. Invest. 57, 625-632.
4. Lieberman, J. and Janoff, A. (1972) in Pulmonary Emphysema and Proteolysis (Mittman, C., ed.), 189-224, Academic Press, Inc., New York.
5. Taylor, J. C. and Crawford, I. P. (1975) Arch. Biochem. Biophys. 169, 91-101.
6. Schmidt, W. and Havemann, K. (1974) Z. Physiol. Chem. 355, 1077-1082.
7. Zimmerman, M. and Ashe, B.M. (1977) Biochim. Biophys. Acta, in press.
8. Quinn, R. S. and Blout, E. R. (1970) Biochem. Biophys. Res. Commun. 40, 328-333.
9. Starkey, P. M. and Barrett, A. J. (1976) Biochem. J. 155, 273-278.
10. Orly, J. and Schramm, M. (1975) Proc. Nat. Acad. Sci. 72, 3433-3437.
11. Erlanger, B. F., Wassermann, N. H., Cooper, A. G., and Monk, R. J. (1976) Eur. J. Biochem. 61, 287-295.