Biochimica et Biophysica Acta, 480 (1977) 241—245

BBA 68016

SUBSTRATE SPECIFICITY OF THE ELASTASE AND THE CHYMOTRYPSIN-LIKE ENZYME OF THE HUMAN GRANULOCYTE

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(Received May 17th, 1976)

Summary

Human granulocyte elastase (EC 3.4.21.11) differs from hog pancreatic elastase in its specificity for synthetic substrates. Although hydrolyzing peptide bonds adjacent to the carboxyl group of alanine, the granulocyte enzyme prefers valine at the cleaved bond, in contrast to the pancreatic enzyme which prefers alanine. Peptide bonds involving the carboxyl group of isoleucine can be hydrolyzed by the granulocyte enzyme but are not hydrolyzed to any significant extent by pancreatic elastase. This difference in specificity could explain the lower sensitivity of the granulocyte enzyme to inhibitors containing alanine analogs, such as the peptide chloromethyl ketones and elastatinal. The human granulocyte chymotrypsin-like enzyme differs from pancreatic chymotrypsin by being able to cleave substrates containing leucine in addition to those containing the aromatic amino acids.

Introduction

The neutral proteases of the human granulocyte are probably involved in the degradation of connective tissue components in inflammatory disease [1]. As a guide to the study of possible inhibitors of these enzymes, we have started to examine the substrate specificity of the major serine proteases of the granulocyte, namely, elastase (EC 3.4.21.11) and the chymotrypsin-like enzyme described by Schmidt and Havemann [2], Feinstein and Janoff [3], and Starkey and Barrett [4].

Human granulocyte elastase has been reported to have a specificity similar to pancreatic elastase, which cleaves peptide bonds involving the carboxyl groups of aliphatic amino acids, particularly alanine [5—7]. The granulocyte elastase hydrolyzes both the natural substrate, elastin, and synthetic substrates such as

Abbreviations: PIPES, piperazine-*N*,*N*'-bis(2-ethane sulfonic acid); TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid.

t-Boc-alanine-*p*-nitrophenyl ester [8] and *N*-acetyl-DL-alanine-α-naphthyl ester [9]. It also, like the pancreatic enzyme, has an extended active site requiring a minimal chain length for a peptide to be a substrate [10]. However, differences in specificity could be inferred from the fact that a tetrapeptide chloromethyl ketone inhibits human granulocyte elastase more slowly than its pancreatic counterpart [10]. In addition, elastatinal, a specific inhibitor of pancreatic elastase [11], is less inhibitory of the granulocyte enzyme than of the pancreatic enzyme (see below and ref. 12). Since the peptide chloromethyl ketone and elastatinal owe their specificity to the fact they both contain alanine analogs, the possibility was considered that the two elastases also differed in their specificity toward synthetic substrates. For this reason, a number of tetrapeptide nitroanilides was prepared by varying the amino acid whose carboxyl group is liberated by enzymatic cleavage of the amide bond. Using these substrates, profound differences in specificity between the two elastases were found.

Recent studies also suggest that the granulocyte chymotrypsin has a substrate specificity and inhibitor response like that of pancreatic chymotrypsin [3,4,13,14]. However, certain differences in susceptibility to chloromethyl ketones have been reported [3]. We therefore tested bovine pancreatic chymotrypsin and the human granulocyte chymotrypsin-like enzyme with the same substrates to determine if such a specificity difference also exists between these enzymes.

Experimental

Materials

Purified human granulocyte elastase was prepared essentially according to the procedure of Taylor and Crawford [15]. Purified human granulocyte chymotrypsin-like enzyme was prepared by a modification of the aforementioned procedure, involving chromatography on CM-Sephadex C-50 according to the method of Schmidt and Havemann [2]. Leukocyte granule extracts from animal bloods were prepared essentially by the procedure of Baugh and Travis [16], but using a Dounce homogenizer to break the cells. Hog pancreatic elastase, chromatographically purified, was obtained from Miles Laboratories. Bovine α-chymotrypsin, three times crystallized, was obtained from Worthington Biochemical Corporation.

Dog, monkey and human pancreas granule extracts were prepared by homogenizing pancreas in 0.35 M sucrose, 0.01 M PIPES * buffer, pH 6.5, with a Teflon pestle homogenizer. The homogenate was centrifuged at 500 × *g* for 10 min and the pellet was discarded. The supernatant was centrifuged at 17 000 × *g* for 10 min and the resulting pellet containing the granules was lysed with Triton X-100. Elastase activity was determined in the crude extract after activation of the zymogen by trypsin. Trypsin does not hydrolyze any of the substrates used in this study.

The chloromethyl ketone analog, Cbz-Ala-Ala-Pro-Ala CH_2Cl, was supplied to us by Dr. James Powers of the Georgia Institute of Technology, and elastatinal was supplied by Dr. T. Aoyagi, Institute of Microbial Chemistry, Tokyo.

† See footnote p. 241.

Methods

Enzymatic assays. Enzyme assays were performed in 0.05 M TES † 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.

Inhibition studies. Elastatinal, previously dissolved in Me_2SO, was added to the substrate 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 to the substrate. The inactivations with the chloromethyl ketone were done at room temperature, pH 7.5, in 0.05 M TES buffer containing 10% Me_2SO.

Peptide synthesis. Ac-Ala-Ala-Pro was prepared essentially according to the procedures used by Thompson and Blout [17]. The amino acid nitroanilides were prepared from the amino acid chloride hydrochlorides synthesized using PCl_5 in CCl_4 [18]. The tetrapeptide nitroanilides were prepared by coupling Ac-Ala-Ala-Pro and the amino acid nitroanilide using isobutylchloroformate [17]. Purity of the peptides was checked by thin-layer chromatography, amino acid analysis, and NMR spectroscopy.

Results and Discussion

Elastases

The inhibition of the elastinolytic enzymes by the tetrapeptide chloromethyl ketone and elastatinal, a specific peptide aldehyde inhibitor of pancreatic elastase [11], is shown in Table I. As reported by Powers [10], the human granulocyte enzyme reacts more slowly with the tetrapeptide chloroketone, Ac-Ala-Ala-Pro-Ala CH_2Cl, than does pancreatic elastase from a number of species. In addition, elastatinal is a poorer inhibitor of the granulocyte elastase than of the

TABLE I

DIFFERENTIAL INHIBITION OF ELASTASES

The substrate was 0.2 mM *t*-Boc-Ala-Ala-Pro-Ala-*p*-nitroanilide in 0.05 M TES buffer (pH 7.5) incubated at 25°C.

Elastase	Chloromethyl ketone (min) *	Elastatinal k_i (M)
Pancreas		
Hog	1	$3 \cdot 10^{-7}$
Human	8	$1 \cdot 10^{-7}$
Dog	n.d. **	$3 \cdot 10^{-7}$
Monkey	5	$2 \cdot 10^{-7}$
Granulocyte		
Human	50	$8 \cdot 10^{-5}$
Dog	n.d.	$1 \cdot 10^{-4}$
Monkey	n.d.	$1 \cdot 10^{-4}$

* Minutes of preincubation at 25°C for complete inactivation with 1 mM inhibitor.

** n.d., not determined.

† See footnote p. 241.

pancreatic elastase, independent of species. This shows that the differential inhibition of the two elastases, also reported by Feinstein and Janoff [12], is not a peculiarity of the human enzyme. Both of these inhibitors owe their specificity to the presence of alanine analogs in the terminal position.

In an attempt to explain this observed differential inhibition, the peptides shown in Table II were synthesized and examined for hydrolysis by the elastases. Activities are expressed relative to the hydrolytic rate with the alanine nitroanilide as substrate. Kinetic constants for those peptides which are good substrates are reported in Table III.

It can be seen that the rates of hydrolysis of the various substrates by the elastases are markedly different. Whereas alanine is the preferred amino acid for the pancreatic elastase, the granulocyte enzyme prefers valine. In addition, the granulocyte elastase hydrolyzes the isoleucyl nitroanilide bond which is relatively resistant to the pancreatic enzyme. Such apparent differences in substrate specificity between the two elastases could easily explain the differential inhibition by alanine-based inhibitors.

A study of the effect of substitutions at amino acids further along the peptide chain from the bond cleaved would be of interest, particularly in the light of the observation of Powers [19] that the position of proline in the peptide chain has an effect on the ability of a chloromethyl ketone-containing peptide to inhibit the two elastases.

Chymotrypsins

The two chymotryptic enzymes differ in their response to some inhibitors but not to others. Feinstein and Janoff [3] reported that whereas *N*-carbobenzoxy-L-phenylethyl chloromethyl ketone (Z-PheCH_2Cl) inhibits both the pancreatic and granulocyte chymotrypsins, L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (Tos-PheCH_2Cl) inhibits only the pancreatic enzyme. However, chymostatin, a peptide aldehyde containing phenylalaninal in the C-terminal position [20], is a potent inhibitor of both enzymes.

The two enzymes also differ in their ability to cleave the substrates studied

TABLE II

HYDROLYSIS OF SYNTHETIC SUBSTRATES BY ELASTASES AND CHYMOTRYPSINS

Substrate: Ac-Ala-Ala-Pro-X-*p*-nitroanilide. Elastases: rates relative to Ala as 100; chymotrypsin: rates relative to Phe as 100.

Enzyme	X					
	Ala	Gly	Val	Leu	Ile	Phe
Elastase						
Hog Pancreas	100	1	5	25	0	0
Human Granulocyte	100	2—20 *	800	20	150	0
Chymotrypsin						
Bovine Pancreas	0	0	0	0	0	100
Human Granulocyte	0	0	0	65	0	100

* For reasons undefined at this time, the glycine derivative gave non-linear kinetics with respect to both substrate and enzyme concentrations.

TABLE III

KINETIC CONSTANTS FOR THE HYDROLYSIS OF SUBSTRATES

Enzyme	Substrate *	K_m (M)	K_{cat} (s^{-1})	$\frac{K_{cat}}{K_m}$
Hog pancreas elastase	Ala	$9.5 \cdot 10^{-4}$	43	45 200
	Val	$1.3 \cdot 10^{-2}$	22	1 700
	Leu	$4.0 \cdot 10^{-3}$	22	5 500
Human granulocyte elastase	Ala	$1.5 \cdot 10^{-3}$	5.9	3 900
	Val	$3.1 \cdot 10^{-4}$	8.1	27 000
	Ile	$1.2 \cdot 10^{-3}$	6.5	5 400
Bovine pancreas chymotrypsin	Phe	$2.3 \cdot 10^{-4}$	9.8	42 600
Human granulocyte chymotrypsin	Phe	$1.3 \cdot 10^{-3}$	0.19	154
	Leu	$1.0 \cdot 10^{-3}$	0.096	96

* X of Ac-Ala-Ala-Pro-X-*p*-nitroanilide.

in this report. From Table II, it can be seen that although both the pancreatic and the granulocyte chymotryptic enzymes prefer the phenylalanine peptide, the granulocyte enzyme also hydrolyzes the leucyl peptide. However, the pancreatic enzyme hydrolyzes these nitroanilide substrates much more readily than does the granulocyte enzyme, with both K_{cat} and K_m contributing to this difference (Table III). The possibility that the sequence of the tetrapeptide used could be unfavorable for hydrolysis by these enzymes will be the subject of future studies.

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