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The Reaction of Active-Site Inhibitors with Elastase Using a New Assay Substrate

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1. Some active-site directed chloroketone derivatives of elastase have been synthesised and characterised. Their inhibition constants and rate constants for inhibition of elastase have been determined.

2. A new spectrophotometric substrate of elastase has been developed.

3. Inhibition of elastase increases in efficiency with length of polypeptide chain except that in *N*-acetylated derivatives the dipeptide is significantly less reactive when bound to the enzyme than is a simple alanine derivative.

Since the determination of the primary and tertiary structure of elastase [1,2] efforts have been made by a number of workers [3—6] to develop an active-site directed irreversible inhibitor for the enzyme. Such inhibitors would be useful in the crystallographic study of substrate binding, in the elimination of elastase activity from that of a mixture of other enzymes, and perhaps pharmacologically since elastase is involved in the production of the symptoms of certain diseases, *e.g.* atherosclerosis and pulmonary emphysema.

Until recently the efforts to find such inhibitors have been unrewarded but recently two groups [5,6] have reported successful inhibition by peptide chloromethyl ketones. These workers were largely concerned with longer-chain peptide derivatives. We have studied short chain *N*-acyl derivatives of L-alanyl chloromethyl ketone, di-L-alanyl chloromethyl ketone and tri-L-alanyl chloromethyl ketone. In this study we have used *N*-benzyloxycarbonyl-L-glutamyl-L-alanine nitrophenyl ester as an assay substrate for elastase.

MATERIALS AND METHODS

Elastase was prepared from pancreatin by the method of Shotton [7] or obtained from Whatman Biochemicals Ltd (Maidstone, England). Both preparations were identical in activity and showed only one band on gel electrophoresis. Enzyme stock solutions were prepared in distilled water, their molarity being determined from the weight of enzyme added.

Abbreviations. Ac-, acetyl; $-CH_2Cl$, chloromethyl; —OMe, methoxyl; —ONp, *para*-nitrophenoxyl; Z—, benzoxycarbonyl.

Enzymes. Elastase (EC 3.4.4.7); chymotrypsin (EC 3.4.4.5); trypsin (EC 3.4.4.4).

SYNTHESIS OF SUBSTRATES AND INHIBITORS

Melting points, specific rotations and elemental analyses are given in Table 1. All compounds gave satisfactory infrared and nuclear magnetic resonance spectra.

Preparation of Ac-Ala-Ala-Ala-OMe

The method followed that for the preparation of the ethyl ester by Goodman and Langsam [8]. The product was recrystallised twice from ethanol.

Preparation of Z-Glu-Ala-ONp

γ-*t*-Butyl *N*-benzyloxycarbonyl-L-glutamate (prepared by the method of Itoh [9]) (1.685 g), isobutyl chloroformate (0.66 ml) and triethylamine (0.69 ml) were added consecutively to dimethyl formamide (15 ml) at —5 °C. The mixture was stirred at —5 °C for 30 min. *p*-Nitrophenyl alaninate hydrobromide (1.455 g) was added and then triethylamine (0.69 ml) in dimethyl formamide (5 ml) was added dropwise over 1 h. The mixture was stirred at room temperature for 4 h, methylene dichloride (50 ml) was added and the solution washed with water, aqueous citric acid and aqueous sodium carbonate. The organic layer was dried over magnesium sulphate and the solvent evaporated. Recrystallisation of the residue from chloroform/hexane gave Z-Glu(OBu^t)-Ala-ONp (1.74 g) as a white powder, m.p. 138—8.5 °C, $[\alpha]_D^{20}$ —38.1° (1, $CHCl_3$).

Table 1. *Physical constants of substrates and inhibitors*

Compound	Formula	m. p.	$[\alpha]_D^{20}$ (concn, solvent)		Analysis C	H	N	Cl
		°C	°		%	%	%	%
Ac-Ala-Ala-Ala-OMe	$C_{12}H_{21}H_3O_5$	254—5	—147.5 (1, water)	Calcd	50.2	7.4	14.6	—
				Found	50.1	7.4	14.8	—
Z-Glu-Ala-ONp	$C_{22}H_{23}N_3O_9$	155—6	— 37.8 (1, EtOAc)	Calcd	55.8	4.9	8.9	—
				Found	55.7	5.0	8.6	—
Z-Ala-CH_2Cl	$C_{12}H_{14}NO_3Cl$	90—1	— 46.0 (2, EtOH)	Calcd	56.4	5.5	5.5	13.8
				Found	56.4	5.7	5.4	13.6
Z-Ala-Ala-CH_2Cl	$C_{15}H_{19}N_2O_4Cl$	139—41	— 71.5 (2, EtOH)	Calcd	55.1	5.9	8.6	10.9
				Found	55.3	6.1	8.9	10.6
Z-Ala-Ala-Ala-CH_2Cl	$C_{18}H_{24}N_3O_5Cl$	152.5—3.5	— 86.3 (1, EtOH)	Calcd	54.3	6.1	10.6	8.9
				Found	54.3	6.1	10.6	9.1
Ac-Ala-CH_2Cl	$C_6H_{10}NO_2Cl$	98—100	— 61.0 (3, water)	Calcd	44.1	6.2	8.6	21.7
				Found	44.0	6.1	8.4	21.5
Ac-Ala-Ala-CH_2Cl	$C_9H_{15}N_2O_3Cl$	145—6	—100.0 (1, water)	Calcd	46.1	6.4	11.9	15.1
				Found	45.9	6.2	11.6	15.0
Ac-Ala-Ala-Ala-CH_2Cl	$C_{12}H_{20}N_3O_4Cl$	180—2	—122.9 (1, water)	Calcd	47.1	6.6	13.7	11.6
				Found	46.8	6.5	13.8	11.6

This product (1.0 g) was added to trifluoroacetic acid (10 ml). After 1 h the trifluoroacetic acid was evaporated under reduced pressure and the residue recrystallised from chloroform giving Z-Glu-Ala-ONp (0.7 g) as pale yellow crystals.

Z-Ala-CH_2Cl

Benzyloxycarbonyl-L-alanine (13.4 g) isobutyl chloroformate (7.93 ml) and triethylamine (8.4 ml) were added consecutively to dry ethyl acetate (50 ml) at —5 °C. The mixture was stirred for 20 min. An ethereal solution of diazomethane (0.12 mol) was added and stirring continued for 1 h at 0 °C. The yellow solution was decolourised with dry hydrogen chloride, washed with aqueous sodium bicarbonate and dried over magnesium sulphate. Evaporation of the solvent gave a yellowish solid which was triturated with ether to give pure Z-Ala-CH_2Cl as a white powder. The material could be further purified by recrystallisation from ethyl acetate/ether.

Alanine chloromethyl ketone hydrobromide was prepared by treatment of Z-Ala-CH_2Cl with an anhydrous solution of hydrogen bromide in acetic acid for 20 min. Dry ether was added to precipitate the hydrobromide and the flask kept at 5 °C for 24 h. The crude product was collected by filtration, washed with ether and dried over potassium hydroxide pellets. It was normally used directly but could be purified by recrystallisation from methanol/ether m.p. 89—91 °C dec.

Z-Ala-Ala-CH_2Cl

The mixed anhydride formed by reaction of benzyloxycarbonyl-L-alanine (4.46 g, 20 mmol) was prepared at —5 °C as above except that tetrahydrofuran was used as the solvent. Alanine chloromethyl ketone hydrobromide (4.08 g, 20 mmol) was added and triethylamine (2.8 ml, 20 mmol) added dropwise over 1 h at 0 °C. The mixture was stirred at room temperature when the product was worked up in the usual manner. Recrystallisation from chloroform/hexane gave Z-Ala-Ala-CH_2Cl (4.2 g, 60%) as white platelets.

Z-Ala-Ala-Ala-CH_2Cl

Benzyloxycarbonyl-L-alanyl-L-alanine was prepared by hydrolysis of the ethyl ester [8] and treated in the same manner as benzyloxycarbonyl-L-alanine above to give Z-Ala-Ala-Ala-CH_2Cl (4.2 g, 50%) as a white powder after two recrystallisations from chloroform.

Ac-Ala-CH_2Cl

Glacial acetic acid and alanine chloromethyl ketone hydrobromide were condensed in tetrahydrofuran solution by the mixed anhydride method above. Ethyl acetate was added and the mixture filtered to remove precipitated triethylamine hydrochloride. Evaporation of the solvent and trituration of the residue with ether gave a powder which gave Ac-Ala-CH_2Cl (25%) as colourless needles after recrystalli-

sation from ethyl acetate. The material was very soluble in water.

Ac-Ala-Ala-CH_2Cl

Acetyl-L-alanine was condensed with alanine chloromethyl ketone hydrobromide by the usual method except that *N*-methylmorpholine was used as the base. The reaction mixture was evaporated to dryness and the residue recrystallised twice from ethyl acetate giving Ac-Ala-Ala-CH_2Cl (20% yield) as a white powder. The losses occurred in the recrystallisations which are necessary to remove the *N*-methylmorpholine salts. Normal extraction methods failed because of the water solubility of the product.

Ac-Ala-Ala-Ala-CH_2Cl

Acetyl-L-alanine and benzyl-L-alaninate hydrochloride were condensed as above giving benzyl-*N*-acetyl-L-alanyl-L-alaninate which gave *N*-acetyl-L-alanyl-L-alanine after hydrogenolysis. The product was condensed with alanine chloromethyl ketone hydrobromide as above and the reaction mixture evaporated to dryness. The residue was extracted with ether in a Soxhlet apparatus to give a mixture of the desired product and salts of *N*-methylmorpholine. This mixture was separated by chromatography on silica gel, the desired product being eluted by chloroform. Recrystallisation from methanol/ethyl acetate gave Ac-Ala-Ala-Ala-CH_2Cl as a white powder.

KINETIC MEASUREMENTS

The concentration of active enzyme in the partially inhibited mixture was determined by rate assay using Z-Glu-Ala-ONp as substrate in phosphate buffer pH 6.04 ($I = 0.05$) containing dioxan (4.8%) at 25 °C. An aliquot (0.15 ml) of a dioxan solution of the substrate (0.04 M) was added to the buffer (3 ml) in a 1-cm quartz cuvette. At a given moment the enzyme solution (0.05 ml) was added to this mixture and the rate of liberation of *p*-nitrophenol was followed at 340 nm using a Unicam SP-700 spectrophotometer fitted with a scale expander.

The hydrolysis of Ac-Ala-Ala-Ala-OMe was followed by a Radiometer pH-stat apparatus consisting of a pH meter PHM26, titrator control TTT11 and an automatic burette and recorder. All such measurements were in presence of 1 mM Tris to stabilise the instrument, the ionic strength was brought to 0.05 by addition of potassium chloride.

Determination of Inhibition Constants

Inhibition constants (K_i) were determined for the inhibition of Ac-Ala-Ala-Ala-OMe by the chloroketones. Initial rate measurements were used in which substrate, inhibitor (in dioxan solution) and enzyme were added rapidly and consecutively to the pre-equiliberated buffer in the pH-stat. Because the initial curve produced by the pH-stat is influenced by instrumental factors the true slope at zero time was determined by backward extrapolation of later points by a least-squares fit for a cubic equation. Inhibition constants were determined from Dixon plots.

In the case of Z-Ala-Ala-Ala-CH_2Cl where inhibition constants were required over a range of pH-values, K_i was determined below pH 7 using Z-Glu-Ala-ONp spectrophotometrically and above pH 7 using Ac-Ala-Ala-Ala-OMe. At pH 7.0 the two methods gave identical results.

Determination of Catalytic Constants

The values of K_m and k_{cat} for the substrates used were determined using initial rate measurements. Substrate concentrations varied from $5 \times K_m$ to $0.1 \times K_m$. The values of velocity and substrate concentration were fitted iteratively to a rectangular hyperbola using the program of Hanson and his coworkers [10].

Rates of Inhibition

A solution of the inhibitor in dioxan (0.05 ml) at a concentration which would give a final concentration of $2\,K_i$ was added to an elastase solution (6 μM) in buffer solution (normally phosphate pH 7.00 $I = 0.1$) at 25 °C. The enzyme activity was assayed at least ten times during each run and these points were spread over at least three half-lives of the reaction. The infinity value corresponded to zero activity. All gave good first-order plots but with some of the slower reactions it was necessary to correct for the spontaneous decay of enzyme activity.

RESULTS

Kinetic Parameters of Substrates

The values determined are given in Table 2. It is apparent that high concentrations of dioxan cause inhibition of the enzyme although there is insufficient evidence at present to determine whether this is a specific effect or whether it arises from a small change in the enzyme conformation. The inhibition, which also arises in the presence of other solvents (*e.g.* acetonitrile) is at present under investigation in this laboratory. At concentrations lower than 3% dioxan in water the inhibition becomes undetectable if Ac-Ala-Ala-Ala-OMe is used as the substrate.

Rates of Inhibition

These are given in Table 3. The effect of pH upon the reaction of elastase with Z-Ala-Ala-Ala-CH_2Cl

Table 2. *Kinetic parameters for elastase substrates*
Determined at 25 °C, ionic strength 0.05

Substrate	pH	Dioxan	k_{cat}	K_m	k_{cat}/K_m
		%	s^{-1}	M	$M^{-1}\,s^{-1}$
Ac-Ala-Ala-Ala-OMe[a]	8.00	0	77	0.51	151000
Ac-Ala-Ala-Ala-OMe	8.00	0	73	0.43	170000
Ac-Ala-Ala-Ala-OMe	8.00	10	61	1.05	58000
Z-Glu-Ala-ONp	6.00	2	9.0	0.40	22500
Z-Glu-Ala-ONp	7.00	2	17.7	0.40	44000

[a] From [17]. All other compounds determined in this work.

Table 3. *Kinetic constants*
Determined for reaction of elastase according to the scheme $E + I \underset{K_i}{\rightleftharpoons} [EI] \xrightarrow{k} EI'$. Measured in phosphate buffer pH 7.0 ($I = 0.1$) containing 10% dioxan at 25 °C. [Elastase] = 6 μM

Inhibitor	K_i	$10^4 \times k$	k/K_i
	mM	s^{-1}	M^{-1}, s^{-1}
Z-Ala-Ala-Ala-CH_2Cl	0.2	4.5	2.3
Z-Ala-Ala-CH_2Cl	0.3	0.48	0.16
Z-Ala-CH_2Cl	2	0.45	0.023
Ac-Ala-Ala-Ala-CH_2Cl	0.4	5.4	1.4
Ac-Ala-Ala-CH_2Cl	2	1.65	0.08
Ac-Ala-CH_2Cl	20	3.15	0.016

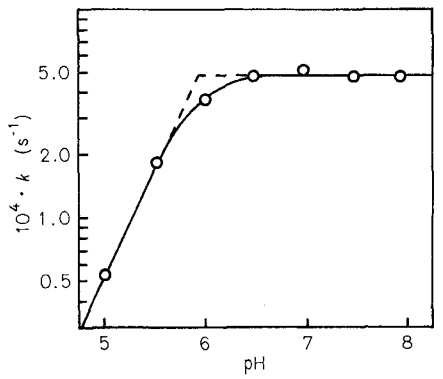

Fig. 1. *The pH-dependence of the reaction of Z-Ala-Ala-Ala-CH_2Cl with elastase.* The rate constants k, determined using the equation $E + I \underset{K_i}{\rightleftharpoons} [EI] \xrightarrow{k} EI'$, are plotted on a logarithmic scale against pH. Reactions were run in 10% aqueous dioxan using acetate buffers below pH 6, phosphate buffers at pH 6 and above ($I = 0.1$)

was studied and the results, with the rate constant plotted on a logarithmic scale, are displayed in Fig. 1. The line plotted in the low-pH region has unit gradient. The pH range available was limited by the spontaneous inactivation of the enzyme whose rate becomes comparable with the inhibition reaction at the limits of the range. The K_i values measured for the inhibitor over this range lie between 0.5 mM at pH 5 and 0.2 mM at pH 8.

DISCUSSION

Z-Glu-Ala-ONp as an Assay Substrate

This substrate was originally developed because a study of the specificity of the action of elastase against the A and B chains of insulin [11] showed that three out of the five main cleavage points occurred at the acyl group of a small residue which was acylated by a negatively charged residue. Whilst not as good a substrate as Ac-Ala-Ala-Ala-OMe in terms of its k_{cat} value, Z-Glu-Ala-ONp is hydrolysed twice as fast, under equivalent conditions, as the other nitrophenyl ester substrate of alanine, *t*-butoxycarbonyl-L-alanine nitrophenyl ester [12]. In addition the negatively charged side-chain makes the glutamyl compound more soluble in aqueous solutions. If the compound binds to the enzyme in the same manner as other substrates then, in the productive mode, the site which normally binds alanine and proline [12] can also be constrained to bind glutamate.

Inhibition Reactions

All six of the inhibitors studied reacted irreversibly with elastase. We have not directly determined the cause of this inhibition but on the basis of previous work with elastase [6] and the closely related enzymes chymotrypsin [14] and trypsin [15] it would seem certain that the inactivation arises from N-alkylation of the active-site histidine residue (His-45) by the chloroketone. This assumption is supported by the study of the effect of pH discussed below.

The inhibition of an enzyme by an active-site directed inhibitor which is a substrate analogue must involve binding of the inhibitor before reaction. This leads to the reaction scheme:

$$E + I \underset{K_i}{\rightleftharpoons} [EI] \xrightarrow{k} EI'$$

where [EI] represents an enzyme · inhibitor complex and EI′ is the covalent compound formed between the inhibitor and the enzyme. The rates of reaction of a series of inhibitors will therefore only be comparable if the true rate constant k is measured, rather than the actual rate of inhibition. The simplest method would have been to have used a concentration of inhibitor considerably greater than K_i but this was impossible for the benzyloxycarbonyl compounds because of their insolubility. The inhibition rates were therefore measured with an inhibitor concentration of 2 K_i for all the inhibitors used and the value of k derived from the observed rate constant by simply multiplying by a factor of 1.5.

The value of k/K_i given in Table 3 is directly comparable with the specificity constant k_{cat}/K_m usually used to interpret substrate specificity. It is apparent that the specificity of the inhibitor increases with increasing length of its peptide chain, which was

also observed by other workers with similar inhibitors [5,6] and indeed is also found for substrate specificity. The increased binding of the benzyloxycarbonyl-substituted inhibitors relative to the acetylated ones probably arises from the hydrophobic nature of the substituent which will lead to the less polar environment within the enzyme being energetically preferred to the aqueous environment outside. There seems to be no evidence for a site specific for phenyl groups.

Plainly in a multiple subsite enzyme like elastase [16] competitive inhibition may arise from inhibitors bound at subsites remote from the active site. Hence a considerable proportion of the inhibition as measured by K_i may represent inhibitor bound in a position where it cannot react at the active site. Thus in a series where the chloroketone is carried by the same terminal residue, variations in the rate constant for inhibition probably reflect the productivity of the binding rather than minor steric effects produced within the active site by the remote changes in the inhibitor. In the acetylated series reported here the dipeptide derivative has a lower rate constant than the tripeptide or simple amino-acid derivatives. Using the subsite terminology of Shotton [16] it therefore seems that a dialanine peptide derivative is less likely to bind with its C-terminal residue in the S_1 subsite than in an alanine derivative. This is presumably because the dipeptide is excluded in some way from the S_2 subsite. The S_2 subsite will accept an alanine residue if sites S_1 and S_3 are both favourably filled but if this is not the case the equilibrium is against the binding of a neutral alanine derivative in this site. There is evidence [13] that this site binds proline residues in preference to alanine residues. The rate constant observed for the benzyloxycarbonyl series indicates that S_4 will accept phenyl residues since the acetyl and benzyloxycarbonyl tripeptide derivatives have similar rate constants for inhibition. However, the sites nearer to the active site must be much less able to accept a phenyl group since the fall off in rate constant on moving to the dipeptide is much more marked than in the acetyl series.

The pH profile for the reaction of Z-Ala-Ala-Ala-CH_2Cl with elastase shows a dependence on the presence in its basic form of a group pK_a 5.9—6.1. This is rather lower than the value of 6.5 measured kinetically for elastase-catalysed ester hydrolysis [3,12]. This difference may be a real one since a better comparison would be the pH dependence for the hydrolysis of amides rather than esters and this does not seem to have been measured. On the other hand the difference in dependence may arise from the presence of dioxan since organic solvents do significantly affect the enzyme's activity. Despite this ambiguity the observed pH dependence supports the assumption that histidine is the group which reacts with the enzyme.

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