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A STUDY OF THE ALKALINE MESENTERICOPEPTIDASE ACTIVE SITE BY MEANS OF PEPTIDE CHLOROMETHYL KETONES

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Summary

The kinetics of inactivation of alkaline mesentericopeptidase was studied using chloromethyl ketone derivatives of amino acids and peptides. It was shown that Tos-LysCH_2Cl and Tos-PheCH_2Cl did not influence the enzyme activity, while the inhibitory effect of Cbz-Ala-Gly-PheCH_2Cl was 35 times that of Cbz-Ala-PheCH_2Cl. The dependence of the pseudo-first order rate constant of the enzyme inactivation by Cbz-Ala-Gly-PheCH_2Cl on pH and temperature indicated that a group with a pK of 6.59 and ΔH_i of 7.7 kcal/mol was the site of the inhibitor's attack. Amino acid analysis of the modified totally inactive enzyme revealed a definite loss of histidine and after performic acid oxidation a recovery of 3-carboxymethyl histidine. The whole set of experimental data is convincing evidence on behalf of a selective alkylation of the N-3 of the active site histidine after treatment with Cbz-Ala-PheCH_2Cl and Cbz-Ala-Gly-Phe-CH_2Cl. Alkaline mesentericopeptidase possesses an extended active site and only a peptide chloromethyl ketone, covering a determined sequence of the enzyme molecule (S_3, S_2, S_1, S_1', S_2', S_3' . . .) is able to provide effective inhibition. The values of the inactivation constant (k_{inact}) for Cbz-Ala-PheCH_2Cl and Cbz-Ala-Gly-PheCH_2Cl are close to the corresponding values reported for subtilisin *Amylosacchariticus*.

Introduction

Alkaline mesentericopeptidase (EC 3.4.21.-) is a serine proteinase, isolated from a strain of *Bacillus Mesentericus* [1]. Data implying an active site histidine have been obtained by different approaches [2—5]. The attempts to modify the active site histidine by means of methyl *p*-nitrobenzene sulphonate failed [6] (our experiment too). In this paper we tried as active-site-directed agents

substrate-like chloromethyl ketones which are well known to perform highly specific alkylations of the active site histidine of various serine proteinases resulting in strong irreversible inhibition. Unlike the proteinases chymotrypsin [7] and trypsin [8] displaying narrow specificity and strongly inhibited by Tos-PheCH_2Cl and Tos-LysCH_2Cl respectively, the alkaline proteinases of subtilisin type, to which mesentericopeptidase is considered to belong, are inhibited only by peptide chloromethyl ketones [9,10]. The reaction of alkaline mesentericopeptidase with 4 chloromethyl ketones of varying chain lengths was studied in order to provide a chemical confirmation of the active site histidine and information on the size and extent of the active site.

Materials and Methods

Crystalline alkaline mesentericopeptidase was prepared by the method previously described in ref. 1. Protein concentration was determined spectrophotometrically at 280 nm ($\epsilon = 3.55 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [11]). The enzyme preparation was 80% pure with respect to active enzyme as determined by spectrophotometric titration of the active sites by *N-trans*-cinnamoylimidazole [12]. Cbz-Ala-PheCH_2Cl and Cbz-Ala-Gly-PheCH_2Cl were kindly donated by Professor K. Morihara. Tos-LysCH_2Cl (Serva), Tos-PheCH_2Cl (Merck) and *N*-acetyl-L-tyrosine ethyl ester (Kock-Light Lab.) were used. *N*-acetyl-L-phenylalanine was syntesized as described in ref. 13, the physical constants being in good agreement with those reported in ref. 13. 3-Carboxymethyl histidine used as reference compound in the identification of the products in the acid hydrolysate of the oxidized inhibitor-treated enzyme, was syntesized as described in ref. 14.

The enzyme modification was performed as follows: The buffered reaction mixture, containing the enzyme ($5 \cdot 10^{-6}$ M) and the inhibitor ($1 \cdot 10^{-4}$ M) in ratio 1 : 20 was incubated at constant temperature. At different intervals aliquots were taken out and the activity retained was followed titrimetrically using *N*-acetyl-L-tyrosine ethyl ester ($6.6 \cdot 10^{-3}$ M) at pH 8.0, 25°C, 0.1 M KCl on a 'Radiometer' pH-stat. Titrations were performed with $5 \cdot 10^{-3}$ M KOH.

The sample for amino acid analysis was prepared as follows: 80 mg alkaline mesentericopeptidase were dissolved in 300 ml 0.05 M Tris-maleate buffer (pH 7.8), containing 0.02 M $CaCl_2$. A 33 ml aliquot of a $2 \cdot 10^{-4}$ M solution of Cbz-Ala-Gly-PheCH_2Cl in dioxane was added, the final concentrations in enzyme and inhibitor being respectively $7 \cdot 10^{-6}$ and $2 \cdot 10^{-5}$ M (ratio enzyme : inhibitor 1 : 3). 3 h incubation at 40°C led to complete inactivation of the enzyme. The solution was dialysed against distilled water at 4°C and lyophilized. The control sample without inhibitor was treated in the same way and the total activity was retained.

Performic acid oxidation of the modified enzyme was performed using Hirs method at 25°c [15].

The enzyme samples were hydrolysed with 6 M HCl in sealed evacuated tubes for 24 h at 110°C. The amino acid analysis were carried out on an 'AAA 881' amino acid analyzer (Czechoslovakia).

Results

The dependence of the enzyme inactivation on the time of incubation in the presence of inhibitor is shown in Fig. 1. Tos-LysCH_2Cl and Tos-PheCH_2Cl did

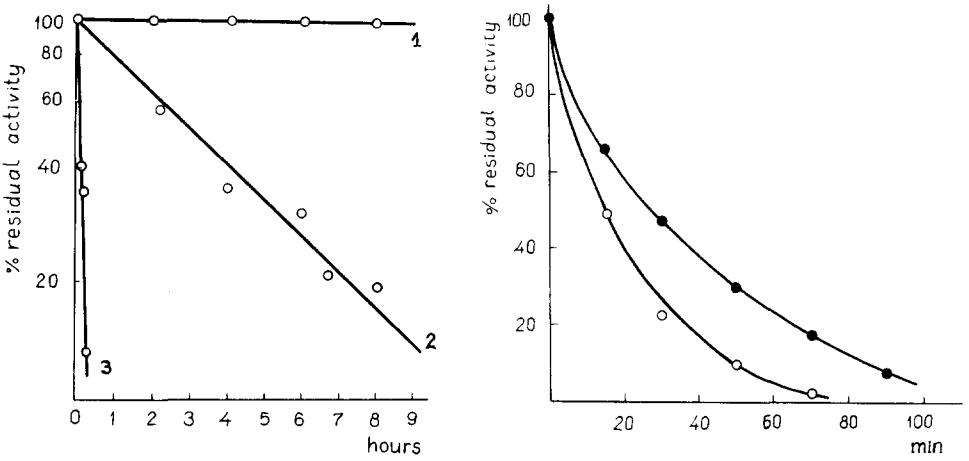

Fig. 1. Inactivation of alkaline mesentericopeptidase by chloromethyl ketones. The enzyme concentration was $5 \cdot 10^{-6}$ M, the inhibitor concentration was $1 \cdot 10^{-4}$ M; 0.05 M Tris-maleate buffer, containing 0.002 M $CaCl_2$ (pH 7.80), 40°C, 10% dioxane (in the case of Tos-LysCH_2Cl-inactivation no dioxane was needed). The residual activity was measured on a pH-stat, using *N*-acetyl-L-tyrosine ethyl ester as substrate. (1) Tos-LysCH_2Cl, Tos-PheCH_2Cl; (2) Cbz-Ala-PheCH_2Cl; (3) Cbz-Ala-Gly-PheCH_2Cl.

Fig. 2. Effect of pH on the pseudo-first order rate constant of Cbz-Ala-Gly-PheCH_2Cl-inactivation of alkaline mesentericopeptidase at 25°C (●) and 40°C (○). Enzyme concentration $5 \cdot 10^{-6}$ M, inhibitor concentration $1 \cdot 10^{-4}$ M, 10% dioxane.

not influence the enzyme activity. 20% of the enzyme activity was retained after 7 h incubation with Cbz-Ala-PheCH_2Cl at 40°C while complete inactivation was achieved in 30 min after treatment with Cbz-Ala-Gly-PheCH_2Cl at the

TABLE I

DEPENDENCE ON pH AND TEMPERATURE OF THE PSEUDO-FIRST ORDER RATE CONSTANT OF Cbz-Ala-Gly-PheCH_2Cl — INACTIVATION OF ALAKALINE MESENTERICOPEPTIDASE

The p*K* values were determined by a least squares treatment of k_{intact} vs. k_{intact} $[H^+]$ dependence.

t (°C)	pH	0.05 M buffer	$k_{intact} \cdot 10^4$ s^{-1}	p*K*
25	5.00	Tris-maleate	0.46	
	5.63	Tris-maleate	1.29	
	6.30	Tris-maleate	6.45	
	6.52	Tris-maleate	8.75	
	7.00	Tris-maleate	14.0	6.59
	7.75	Tris-maleate	17.3	
	8.18	Tris-maleate	18.0	
	8.60	Glycine-NaOH	18.0	
	9.10	Glycine-NaOH	18.2	
40	4.94	Tris-maleate	0.92	
	5.50	Tris-maleate	2.80	
	6.45	Tris-maleate	11.2	
	6.90	Tris-maleate	17.2	
	7.63	Tris-maleate	21.9	6.35
	8.45	Tris-maleate	24.0	
	8.52	Glycine-NaOH	25.2	
	9.00	Glycine-NaOH	24.5	

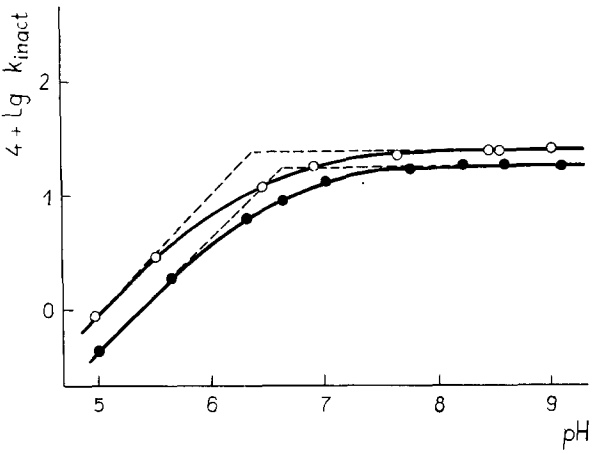

Fig. 3. Inactivation of alkaline mesentericopeptidase by Cbz-Ala-Gly-PheCH$_2$Cl in the presence of the competitive inhibitor *N*-acetyl-L-phenylalanine. Alkaline mesentericopeptidase $5 \cdot 10^{-6}$ M, Cbz-Ala-Gly-PheCH$_2$Cl $6.6 \cdot 10^{-5}$ M, *N*-acetyl-L-phenylalanine $8 \cdot 10^{-3}$ M, 25°C, pH 7.0 (Tris-maleate buffer, containing 0.002 M $CaCl_2$). ●, in the presence, ○, in the absence of *N*-acetyl-L-phenylalanine.

same temperature. From the slope of the plots on Fig. 1 pseudo-first order rate constants of the enzyme inactivation by means of Cbz-Ala-PheCH$_2$Cl ($6.4 \cdot 10^{-5}$ s^{-1}) and Cbz-Ala-Gly-PheCH$_2$Cl ($2.29 \cdot 10^{-3}$ s^{-1}) were determined. Fig. 2 represents the dependence of the rate constant of inactivation of alkaline mesentericopeptidase by Cbz-Ala-Gly-PheCH$_2$Cl on pH and temperature. The corresponding k_{inact} values at different pH and temperature are presented in Table I. The heat of ionization ΔH_i 7.7 kcal/mol was estimated from the pK values at two temperatures: 25 and 40°C, respectively.

N-acetyl-L-phenylalanine, a competitive inhibitor of alkaline mesenterico-

TABLE II

AMINO ACID COMPOSITION OF INACTIVATED AND NATIVE ENZYME

Amino acid	Number of residues	
	Cbz-Ala-Gly-PheCH$_2$Cl-inactivated enzyme	Native enzyme
Lysine	7.0	7.0
Histidine	4.2	5.0
Arginine	3.5	3.8
Aspartic acid	22.1	22.1
Threonine	16.8	17.2
Serine	30.2	30.0
Glutamic acid	13.2	13.4
Proline	10.5	10.8
Glycine	29.7	29.0
Alanine	30.4	30.2
Valine	19.9	19.8
Methionine	3.4	3.4
Isoleucine	12.0	12.5
Leucine	13.6	13.6
Tyrosine	10.8	10.8
Phenylalanine	3.0	3.0

TABLE III

PSEUDO-FIRST ORDER INACTIVATION CONSTANTS FOR SUBTILISINS [10] AND ALKALINE MESENTERICOPEPTIDASE

40°C, pH 7.0, enzyme $5 \cdot 10^{-6}$ M, inhibitor $1 \cdot 10^{-4}$ M, 10% dioxane.

Enzyme	$k_{intact} \cdot 10^5$ (s^{-1})	
	Cbz-Ala-PheCH_2Cl	Cbz-Ala-Gly-PheCH_2Cl
BPN'	9.3	311
Novo	11.9	367
Carlsberg	26.8	284
Amylosaccharitícus Fukumoto	4.2	167
SO4	4.5	184
Alkaline mesentericopeptidase	4.9	175

peptidase ($K_i = 4.5 \cdot 10^{-2}$ M) was shown to have a protective effect towards the irreversible inactivation by Cbz-Ala-Gly-PheCH_2Cl (Fig. 3). This fact also indicates that the loss of activity by Cbz-Ala-Gly-PheCH_2Cl was due to a reaction at the active site of the enzyme. An equivalent amount of 3-carboxymethyl histidine was recovered after performic acid oxidation of peptide chloromethyl ketone-treated urokinase [16], indicating alkylation at *N*-3 of a histidine residue. The same procedure was applied for alkaline mesentericopeptidase. Amino acid analysis of mesentericopeptidase treated with Cbz-Ala-Gly-Phe-CH_2Cl shows a loss of a histidine moiety (Table II). In the acid hydrolysate of the oxidized modified enzyme the peak of 3-carboxymethylhistidine was identified using an authentic sample. The latter was very close to the peak, corresponding to alanine. For this reason and also as a result of the high alanine vs. carboxymethyl histidine ratio (30 : 1) in the mixture, the quantitative determination of 3-carboxymethyl histidine failed. The elution buffer was additionally acidified in an attempt to improve the resolution of both amino acids (alanine and 3-carboxymethyl histidine). As a result the peak corresponding to 3-carboxymethyl histidine became wider, thus reducing its qualitative and quantitative accuracy.

Discussion

Data concerning the involvement of histidine in the active site of alkaline mesentericopeptidase have been reported previously [2—5]. Convincing evidence for this statement is the selective chemical modification of the active site histidine by means of substrate-like peptide chloromethyl ketones, and the detection of the carboxymethyl histidine in the acid hydrolysate of the modified enzyme, which are reported in the present paper. Further evidence bearing on this question is the good agreement between the determined values of the heat of ionization for the process of inactivation with Cbz-Ala-Gly-PheCH_2Cl (ΔH_i = 7.7 kcal/mol), for the hydrolysis of *N*-acetyl-L-phenylalanine methyl ester (ΔH_i = 7.8 kcal/mol) [4] and the value reported for the imidazole residue in proteins (ΔH_i = 6.9—7.5 kcal/mol) [17].

In the presence of the competitive inhibitor *N*-acetyl-L-phenylalanine the

enzyme inactivation by Cbz-Ala-Gly-PheCH$_2$Cl is retarded to some degree, due to the protection of the active site. The protective effect of *N*-acetyl-L-phenylalanine is not significant but is of the same order of magnitude as the effect of hydrocinnamic acid in the case of inactivation of subtilisin BPN′ by the same chloromethyl ketone derivative [9]. Both the competitive inhibitors *N*-acetyl-L-phenylalanine and hydrocinnamic acid are not good inhibitors for these proteinases ($K_i = 10^{-2}$ M). A more obvious protective effect could be expected in the presence of the corresponding tripeptide acid, which should be a better inhibitor for this type of proteinases.

The results we have obtained show that alkaline mesentericopeptidase similarly to subtilisins [18] possesses an extended active site. The catalytic constant increases from $k_{cat} = 200$ s^{-1} to $k_{cat} = 1350$ s^{-1} with elongation of the side chain from *N*-acetyl-L-phenylalanine methyl ester to the dipeptide substrate *N*-acetyl-L-alanyl-L-phenylalanine methyl ester [19]. The inhibitory effect of the chloromethyl ketones studied is dependent on their side chain structure also. Thus the inhibition constant obtained for Cbz-Ala-Gly-PheCH$_2$Cl is 35 times the corresponding one for Cbz-Ala-PheCH$_2$Cl. It is obvious that the elongation of the peptide chain significantly improved the degree of inhibition. This is due to the 'secondary specificity' phenomenon, a characteristic general feature of subtilisins and other alkaline bacterial proteinases [20]. The specificity and nature of substrate-binding centre of such proteinases is discussed generally in terms of nomenclature introduced by Schechter and Berger [21]. According to this system the considerably large binding region of these proteinases can be divided into subsites S_3, S_2, S_1—S_1', S_2', S_3' . . ., each accomodating an appropriate amino acid residue P_3, P_2, P_1—P_1', P_2', P_3' . . . of the peptide substrate, so that the peptide bond cleaved, P_1—P_1', reaches its favourable orientation. Previous studies on the primary specificity of alkaline mesentericopeptidase have shown S_1 to be a hydrophobic cavity to which tyrosine, phenylalanine or leucine in the P_1 position fitted the best [22,23]. The nature of the side chain groups at the P_2 position (alanyl or glycyl) and P_3 position (alanyl) permits us to suppose that the S_2 and S_3 subsites of the enzyme should be of a hydrophobic nature, too. Additional confirmation of this suggestion should be obtained by studying the hydrolysis of a series of peptide ester substrates.

The comparison of the values of the inactivation constants (k_{inact}) for Cbz-Ala-PheCH$_2$Cl and Cbz-Ala-Gly-PheCH$_2$Cl and alkaline mesentericopeptidase on the one hand and subtilisins on the other (Table III) implies the close resemblence between the subtilisin *Amylosacchariticus* and the enzyme studied.

A number of properties such as pH optimum of proteolysis [24], molecular weight [25], amino acid composition [25], topography of amino acid residues [26], kinetic constants for ester substrates [22,23] show a significant similarity between alkaline mesentericopeptidase and the subtilisins without however, being identical with any of the former enzymes.

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PheCH$_2$Cl and to Dr. K. Voivodov for his help and advice in amino acid analysis experiments.

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