INHIBITION OF *STREPTOMYCES GRISEUS* PROTEASE B BY PEPTIDE CHLOROMETHYL KETONES: PARTIAL MAPPING OF THE BINDING SITE AND IDENTIFICATION OF THE REACTIVE RESIDUE

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Received 5 April 1974

1. Introduction

One of the major proteolytic enzymes of Pronase is *Streptomyces griseus* Protease B (SGPB) [1]. This enzyme has been studied by several groups and designated by different names. It seems that SGPB is identical with PNPA hydrolase II [2], alkaline proteinase C [3], Pronase elastase [4], elastase-like enzyme III [5] and guanidine stable endopeptinase B [6,7].

The molecular weight of SGPB has been estimated as 17 500 by gel-filtration [6] and SDS gel electrophoresis [8]. It contains two methionine residues and can be separated into three fragments by cyanogen bromide cleavage and gel chromatography on Sephadex G-50 [9]. It hydrolyzes typical chymotrypsin- and elastase-substrates such as *N*-acetyl-L-tyrosine ethyl ester, *N*-acetyl-L- alanyl -L-alanyl-L-alanine methyl ester [5], *N*-benzoyl-L-leucine ethyl ester and *N*-benzoyl-L-tyrosine ethyl ester [10]. The specificity of SGPB, which has been studied [10], resembles mainly that of chymotrypsin.

The present report describes the reaction of SGPB with different peptide chloromethyl ketones and presents a partial mapping of the binding site. The reactive site has been identified as histidine-33 which is homologous to the active site histidine-57 of bovine chymotrypsin. The pH dependence of the reaction has also been studied and it has been found to be dependent on two single groups with apparent *pKs* of the 6.6 and 9.5.

2. Materials and methods

2.1. *Materials*

Streptomyces griseus protease B (SGPB) was isolated from Pronase (California Research Corporation) by ion exchange chromatography on CM-Sephadex as described by Jurasek et al. [1].

The inhibitors, all of them derivatives of phenylalanine chloromethyl ketone, namely: acetyl-L-alanyl-L-phenylanine chloromethyl ketone (AcAlaPheCK), acetyl-L-leucyl-L-phenylalanine chloromethyl ketone (AcLeuPheCK), acetyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone (AcAlaGlyPheCK), *N*-tert-butoxycarbonyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone (BocAlaGlyPheCK) and *N*-tert-butoxycarbonyl-glycyl-L-leucyl-L-phenylalanine chloromethyl ketone (BocGlyLeuPheCK), were a gift from Dr. K. Kurachi. Their synthesis and characterization have been described previously [11,12]. Tosyl-L-phenylalanine chloromethyl ketone (TosPheCK) was purchased from Cyclo Chemical Co.

2.2. *Inhibition SPGB by peptide chloromethyl ketones*

The reaction mixtures (1.0 ml) were prepared by adding 0.05 ml of inhibitor (0.8 mg/ml in dioxane) to 0.95 ml enzyme (0.5 mg/ml in 0.05 M Tris–HCl buffer pH 8.0). The inhibition was carried out at 25°C. At various time intervals, aliquots were remo-

* Initial part of this work was carried out in the Department of Biochemistry, University of Washington, Seattle, Wash., USA.

ved from the inhibition mixture and assayed immediately for the residual enzymatic activity. The assays were carried out at pH 8.0, 30°C, using Radiometer pH meter 26 equipped with Titrator 11 using 0.02 M *N*-acetyl-L-tyrosine ethyl ester as a substrate. Second order rate constants of inhibition were calculated from semilogarithmic plots.

The pH dependence of inhibition was studied with AcLeuPheCK at the pH range of 5.0–10.8 using 0.05 M Na-maleate (pH 5.0–7.0), Tris–HCl (pH 6.8–9.2) and Na-glycinate (pH 8.2–10.8). In all cases control experiments without inhibitor were carried out simultaneously.

2.3. *Identification of the site of inhibition*

Twenty mg of SGPB were dissolved in 2.2 ml of 0.05 M Tris–HCl buffer pH 8.0 and mixed with 1 mg AcGlyLeuPheCK dissolved in 0.3 ml of dioxane. After 10 min, when more than 95% inactivation was achieved, the enzyme was desalted on a Sephadex G-15 column (1.5 × 25 cm) equilibrated with 1 mM HCl and then lyophylized. One mg of the dried material was removed for amino acid analysis and the rest was dissolved in 1.5 ml 70% formic acid that contained 30 mg cyanogen bromide, incubated for 24 hr in dark at room temperature and lyophylized. The dried material was then dissolved in 1.5 ml 30% formic acid and applied to a Sephadex G-75 column (1.8–50 cm) previously equilibrated with 10% formic acid. The column was developed at room temperature at the rate of 8 ml/hr and 1.4 ml fractions were collected. The digest was separated into two main fractions (see fig. 1, tubes 41–45 and 49–54) that were pooled, dried and analyzed for amino acid composition. The samples were hydrolyzed in vacuo in constant boiling HCl, at 110°C for 22 hr and the amino acid analysis was performed on a BC-200 Biocal Amino Acid Analyzer by a single column method.

A control experiment, in which AcGlyLeuPheCK was omitted, was carried out simultaneously in an identical manner.

3. Results and discussion

3.1. *Inhibition of SGPB by different peptide chloromethyl ketones*

Although the total number of the inhibitors was

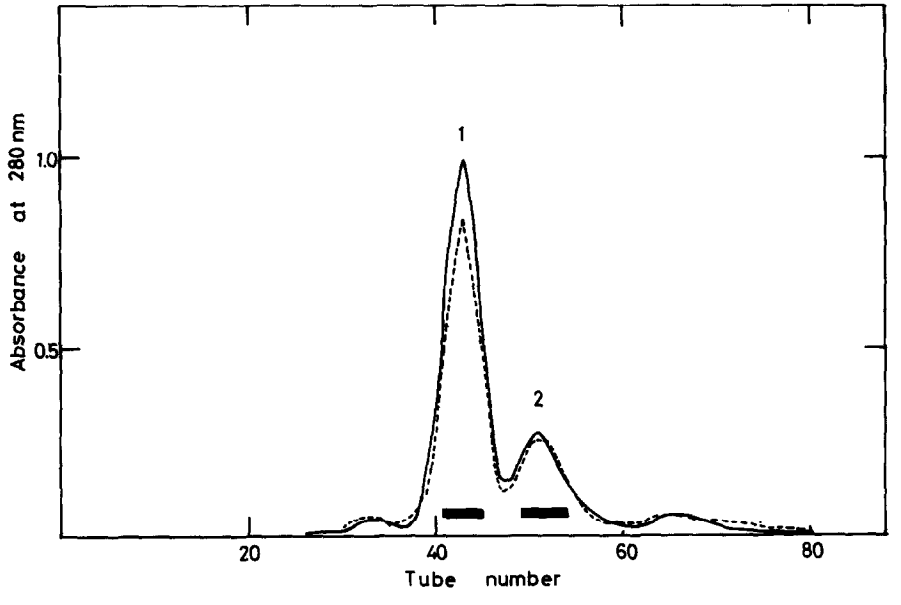

Fig. 1. Separation of cyanogen bromide fragments of 18 mg native (——) and 15 mg of BocGlyLeuPheCK inhibited (- - -) SGPB on Sephadex G-75 column (1.8 × 50 cm) in 10% formic acid. The column was developed at 8 ml/hr and 1.4 ml fractions were collected. (▬) fractions pooled for amino acid analysis.

Table 1
Inhibition of SGPB by various peptide chloromethyl ketones

Inhibitor	k_{II}[a] Of inhibition (min^{-1} M^{-1})
TosPheCK	No inhibition
AcAlaPheCK	7.7 ± 1.6
AcLeuPheCK	595.0 ± 45
AcAlaGlyPheCK	No inhibition
BocAlaGlyPheCK	49.0 ± 9
BocGlyLeuPheCK	4950 ± 1040

[a] Second order rate constant.

quite limited the second order rate constant of inhibition (table 1) indicates clearly that the binding site of SGPB is much more extended than that of bovine chymotrypsin [11,12]. Adopting the notation proposed by Schechter and Berger [13] for the subsites of the binding sites of proteolytic enzymes it can be seen that the subsite S_2 in SGPB requires a relatively large hydrophobic side chain of P_2. The hydrophobic side chain P_1 alone does not satisfy the binding requirements since neither TosPheCK nor AcAlaGlyPheCK inhibit the enzyme. However, when leucyl side chain in P_2 replaces that of glycyl or alanyl the second rate constant of the inhibition increases almost 100-fold, while the corresponding difference in chymotrypsin is only 3–4 times. Although the present data are not sufficient for quantitative evaluation of the importance of P_3 residues in the substrates they indicate strongly that P_4 is also important for optimal binding. For instance BocGlyLeuPheCK is ~7 times more reactive than AcLeuPheCK and BocAlaGlyPheCK inhibits the enzyme while AcAlaGlyPheCK does not.

It may be therefore concluded that the binding site of SPGB consists most probably of a hydrophobic pocket, similarly to that of chymotrypsin but of much wider capacity. This conclusion is further supported by the fact that AcTyrOEt, a typical chymotrypsin substrate, has relatively low affinity for SPGB (K_m = 17.6 mM) while $AcAla_3OMe$, an extended substrate of elastase, binds much stronger (K_m = 2.54 mM) [5].

3.2. *Identification of the inhibition site*

Cyanogen bromide cleavage of SGPB yields three fragments [9]: the N-terminal fragment (No. 1) that consists of ~ 90 amino acids and contains one disulfide bridge and one histidine residue in position 33, the C terminal fragment (No. 2) that consists of ~ 57 amino acids, contains one disulfide bridge and no histidine and the internal fragment (No. 3) that consists of ~ 33 amino acids, has no disulfide bridges and one histidine in position 12 [8]. The N-terminal fragment can be easily separated from the mixture of the internal and C-terminal fragments by gel chromatography on Sephadex G-75 [8]. SPGB was therefore inhibited by BocGlyLeuPheCK, digested by cyanogen bromide and separated into two main fractions (fig. 1). Amino acid composition of the inhibited enzyme indicated that ~ 0.8 histidine residue was destroyed and as expected corresponding amounts of leucine and glycine were incorporated (table 2). Amino acid composition of the separated fragments shows clearly that the reactive histidine is located in the N-terminal fragment (table 2). The amino acid sequence near this histidine is $Thr_{30}Ala_{31}Gly_{32}His_{33}Cys_{34}$ [8] and indicates strong homology to the surroundings of active site histidine in the chymotrypsin family [14].

3.3. *The pH dependence of the inhibition*

Fig. 2 shows clearly that the pH profile of inhibition of SGPB by AcLeuPheCK yields a bell-shaped curve which is dependent on ionization of two single groups. One of these groups with $pK \sim 6.6$ is most

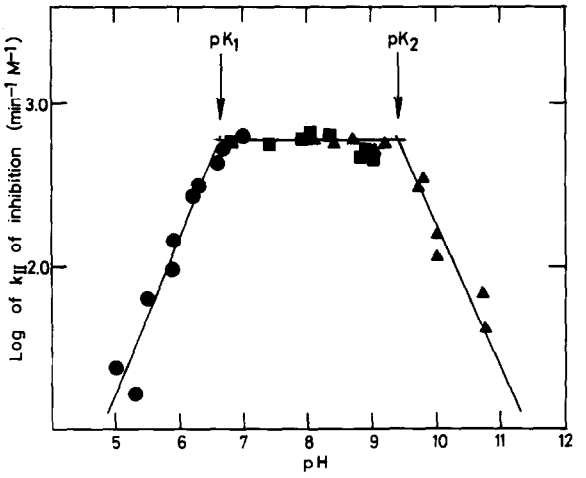

Fig. 2. The pH dependence of k_{II} (second order rate constant) of inhibition of SGPB by AcLeuPheCK (1.14 mM) in 0.05 M Na-maleate (•), Tris–HCl (▪) and Na-glycinate (▲).

Table 2
Amino acid composition of native and BocGlyLeuPheCK-inhibited SGPB and its CNBr fragments. The results were calculated assuming 15 residues of alanine per mole of the whole enzyme, 8 residues in CNBr fragments 1 and 7 residues in CNBr fragments 2 + 3

Amino acids	Whole enzyme		Peak no. 1 (CNBr fragment 1)		Peak no. 2 (CNBr fragments 2 + 3)	
	Native	Inhibited	Native	Inhibited	Native	Inhibited
Aspartic acid	16.21	15.84	10.32	10.26	5.78	6.20
Threonine	24.03	23.81	13.90	13.50	10.64	10.08
Serine	19.10	18.70	9.45	9.57	9.62	9.35
Glutamic acid	4.95	4.67	1.74[a]	2.01[a]	3.95[a]	4.20[a]
Proline	5.30	4.97	2.88	3.21	3.30	3.65
Glycine	30.32	31.26	14.12	14.75	16.82	17.22
Alanine	15.00	15.00	8.00	8.00	7.00	7.00
Half cystine	4.06	3.88	1.34	1.42	1.46	1.68
Valine	12.43	12.04	5.04	4.95	7.50	7.70
Methionine	1.96	1.93	0.02	0.05	0.04	0.03
Isoleucine	6.11	6.34	4.30	4.32	2.17	2.21
Leucine	7.09	7.75	3.20	4.08	4.22	4.30
Tyrosine	9.04	8.96	4.77	4.69	4.35	4.30
Phenylalanine	4.62	4.57	2.78	2.80	2.11	2.15
Lysine	1.15	1.25	0.93	1.08	0.10	0.20
Histidine	2.04	1.18	1.01	0.24	1.10	0.98
Arginine	7.63	7.87	3.86	3.97	3.89	3.72

[a] Including homoserine.

likely histidine 33, as noted before, while the other group with p$K \sim 9.5$ could not be identified. It should be noted that in the pancreatic serine proteases like trypsin [15–17], chymotrypsin [18] and elastase [19–21] this group was identified as the alpha amino group of the N-terminal amino acid that forms an ion pair with the beta-carboxyl group of Asp-194 (according to the numbering system of chymotrypsinogen A) [14].

Although the GlyAspSerGly sequence around the active site serine exists also in SPGB [8], further evidence should be provided in order to find out whether a similar ion-pair exists also in this enzyme and how it effects its enzymatic activity.

Acknowledgements

I wish to thank Dr. K. Kurachi from the University of Washington in Seattle for the peptide chloromethyl ketones and to Miss Geta Tinman for technical assistance.

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