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INACTIVATION OF PYROGLUTAMYL AMINOPEPTIDASE BY L-PYROGLUTAMYL CHLOROMETHYL KETONE

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Summary

A chloromethyl ketone derivative of pyroglutamic acid was newly synthesized and its reactivity with bacterial pyroglutamyl aminopeptidase (L-pyroglutamyl-peptide hydrolase, EC 3.4.11.8) as an affinity labelling reagent was examined. The compound was found to inactivate the enzyme markedly and rapidly at very low concentrations, though the enzyme was resistant to *N*-tosylphenylalanyl chloromethyl ketone. The rate of the enzyme inactivation by pyroglutamyl chloromethyl ketone was retarded in the presence of a poor substrate, pyroglutamyl valine. The enzyme inactivated by treating with *p*-chloromercuribenzoate failed to react with pyroglutamyl chloromethyl ketone. These results strongly suggest an active site-directed mechanism for the enzyme inactivation by pyroglutamyl chloromethyl ketone. This compound was shown to be useful as a titrant for the catalytically active protein of pyroglutamyl aminopeptidase.

Introduction

Pyroglutamyl aminopeptidase (L-pyroglutamyl-peptide hydrolase, EC 3.4.11.8) has been reported to be widely distributed in various bacteria [1—5], plants and animal tissues [6—10]. Most of these reports show that the enzyme is quite sensitive to several sulfhydryl group-blocking reagents and heavy metal ions, suggesting the involvement of a thiol group in the enzyme reaction. How-

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Abbreviations: PGCK, pyroglutamyl chloromethyl ketone; Z-Pyr, benzyloxycarbonyl pyroglutamic acid; Pyr-2-NNap, pyroglutamyl β-naphthylamide.

ever, detailed information on the active site of the enzyme is not yet available.

Recently, we purified pyroglutamyl aminopeptidase from *Bacillus amyloliquefaciens* and characterized its enzymatic properties and substrate specificity [5,11,12]. This bacterial enzyme was also very sensitive to sulfhydryl group-blocking reagents, and stabilized by 2-mercaptoethanol and metal chelating reagents [5].

It has been reported that cysteine residue in the active site of papain, ficin and bromelain is alkylated stoichiometrically with chloromethyl ketone derivatives of *N*-tosyl-phenylalanine and *N*-tosyl-lysine, and that this treatment resulted in complete loss of the enzyme activity [13—15]. Another thiol protease, clostripain, was also shown to be irreversibly inactivated by *N*-tosyl-lysyl chloromethyl ketone.

In order to obtain some information on the active site of pyroglutamyl aminopeptidase from *B. amyloliquefaciens*, we examined the reactivity of pyroglutamyl chloromethyl ketone with the enzyme, suspecting that this chloromethyl ketone derivative behaves as a specific affinity reagent for the enzyme. In this communication we report the effect of this new chloromethyl ketone derivative on the activity of pyroglutamyl aminopeptidase from *B. amyloliquefaciens*.

Materials and Methods

Enzymes. Pyroglutamyl aminopeptidase from *B. amyloliquefaciens* was purified to homogeneity by the method previously described [5] and the enzyme activity was followed by measuring spectrofluorimetrically the initial rate of hydrolysis of pyroglutamyl β-naphthylamide (Pyr-2-NNap), at 30°C in a volume of 2.3 ml, containing varied concentrations of the enzyme/2 mM Pyr-2-NNap/0.12 mM 2-mercaptoethanol/0.1 M phosphate buffer, pH 6.0. The fluorescence of β-naphthylamine liberated was monitored continuously with a Hitachi 512 spectrofluorimeter at wavelengths of 317 nm for excitation and 410 nm for emission. Crystalline preparations of bovine pancreatic trypsin, α-chymotrypsin and papain were purchased from Sigma Chemical Co., U.S.A. The activities were assayed using ethyl *N*-benzoyl-arginine [17], *p*-nitrophenyl-acetate [18] and carbobenzoxy glycyl *p*-nitrophenyl ester [19] as substrates, respectively. Proteins were determined by the method of Lowry et al. [20] using bovine serum albumin as standard, and the concentration of pyroglutamyl aminopeptidase was calculated using a value of 72 000 as its molecular weight [5].

Chemicals. *N*-Tosyl phenylalanyl chloromethyl ketone (TPCK), phenylmethanesulfonyl fluoride and substrates for trypsin, α-chymotrypsin and papain were obtained from Sigma Chemical Co., U.S.A. Benzyloxycarbonyl pyroglutamic acid (Z-Pyr) was purchased from Protein Research Foundation, Minoh, Japan. *N*-Methylmorpholine, isobutylchloroformate, *p*-chloromercuribenzoate (PCMB) and 2-mercaptoethanol were products of Nakarai Chemical Co., Kyoto, Japan. All optically active amino acids and their derivatives used here were of L-configuration, unless otherwise specified.

Synthesis of pyroglutamyl chloromethyl ketone (PGCK). Z-Pyr was coupled with diazomethane according to the mixed anhydride method described by

Anderson et al. [21]. A solution containing 2.56 g (1.0 mmol) Z-pyr and 1.02 ml *N*-methylmorpholine in 15 ml dry ethyl acetate was cooled at −4°C and mixed with 1.18 ml isobutyl chloroformate with vigorous stirring, After 5 min, dry ethereal diazomethane was further added and the reaction mixture was maintained with stirring at −4°C for 30 min, followed by incubation at room temperature for 2 h and final treatment with dry hydrogen chloride at room temperature for 2 h. The solvent was removed by evaporation and the residue, Z-Pyr chloromethyl ketone, was crystallized from ethanol: Yield, 2.51 g (85%); m.p., 154—156°C.

Z-Pyr chloromethyl ketone was further treated with trifluoroacetic acid at 65°C for 5 min. After removal of trifluoroacetic acid by evaporation in vacuo, the residue was dissolved in chloroform and subjected to Silica Gel column chromatography at 4°C using ethyl acetate as an eluent solvent. The ethyl acetate fraction containing the desired PGCK was concentrated by evaporation. The residual oily substance was triturated with hexane to yield a product (0.27 g, 20%) which gave a single spot when chromatographed on a Silica Gel 60 F-254 plate.

Pyr-2-NNap was synthesized by the method described by Szewczuk and Mulczyk [2].

Results

Reaction of PGCK with pyroglutamyl aminopeptidase. At an approx. 3-fold molar excess of PGCK to enzyme protein concentration, the activity of pyroglutamyl aminopeptidase was inhibited rapidly over 95% of the initial level, within 1 min (Fig. 1), and we failed to determine the rate constant of the inhibition because it was impossible to obtain the reliable value due to the extreme rapidity of the reaction.

The addition of 10 mM 2-mercaptoethanol to PGCK-inactivated enzyme did not restore any activity, while PCMB-inactivated enzyme could be reactivated by subsequent treatment with 2-mercaptoethanol. If PGCK reacts with the thiol group of the enzyme, PCMB-inactivated enzyme could be protected from the reaction with PGCK and its activity restored after dialysis and incubation with 2-mercaptoethanol. The experiments were carried out to examine whether this assumption was correct or not, and the results obtained were fully satisfac-

TABLE I

COMPARATIVE REACTIVITY OF SEVERAL ALKYLATING REAGENTS WITH PYROGLUTAMYL AMINOPEPTIDASE

Reaction was carried out at 30°C in 0.1 M phosphate buffer, pH 6.0/14 nM enzyme/0.12 mM 2-mercaptoethanol. Relative $I_{95\%}$ is defined as the molar concentration of reagent required to produce 95% inhibition in 10 min, relative to PGCK.

Reagent	Relative $I_{95\%}$
Pyroglutamyl chloromethyl ketone	1.0
N-Tosyl-phenylalanyl chloromethyl ketone	7.9×10^4
Phenylmethanesulfonyl fluoride	4.8×10^5
Iodoacetic acid	5.7×10^3

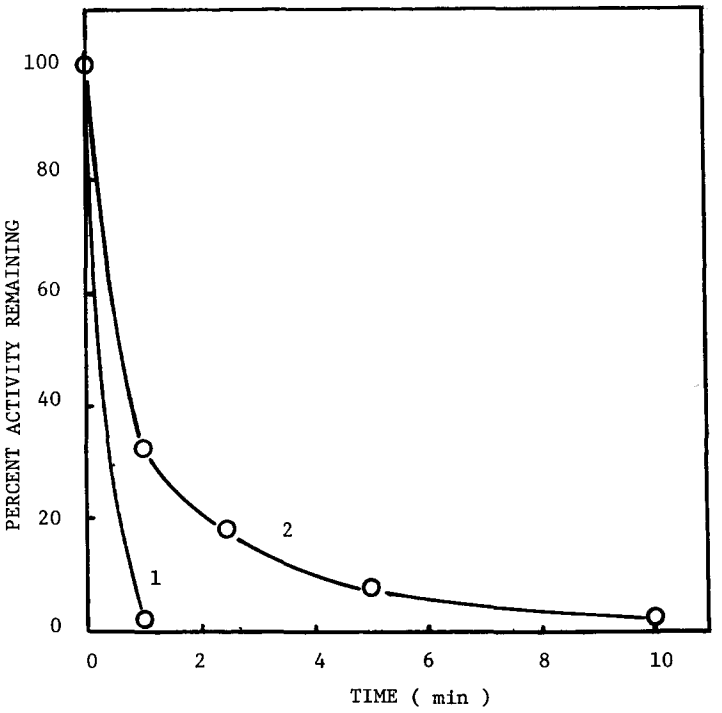

Fig. 1. Inhibtion of pyroglutamyl aminopeptidase by PGCK. Reaction was carried out at 30°C in 0.1 M phosphate buffer, pH 6.0/0.12 mM 2-mercaptoethanol/14 nM enzyme. The concentrations of PGCK were: 1, 45 nM; 2, 10 nM.

tory; namely, PCMB-inactivated enzyme, even if treated with a great excess of PGCK, fully restored its activity after dialysis and reduction with 2-mercaptoethanol.

Table I compares the inhibitory effect of various alkylating reagents on pyroglutamyl aminopeptidase, on the basis of the molar concentration of these reagents required to give 95% inhibition in 10 min, relative to PGCK. These results indicate a remarkable restricted specificity of PGCK, even when compared with the chemically more reactive iodoacetate.

Effect of a poor substrate on inactivation of pyroglutamyl aminopeptidase by PGCK. We previously reported that Pyr-Val was the substrate with higher affinity to the enzyme (K_m: 0.09 mM) but was split at a rather slow rate among 20 pyroglutamyl amino acids tested [11]. As shown in Fig. 2, Pyr-Val protected the enzyme from the inactivation by PGCK markedly while increasing its concentration. 40% of the activity of the control still remained after 10 min incubation with PGCK, in the presence of 0.58 mM Pyr-Val, while almost complete loss of the activity was observed in the absence of Pyr-Val. This result provides strong evidence that PGCK reacts specifically at the active site of the enzyme to bring about inhibition.

The effect of PGCK on the other proteases was also examined. No appreciable inactivation of papain, trypsin and α-chymotrypsin was observed under the condition of relatively high concentration of PGCK and longer incubation periods (data not shown).

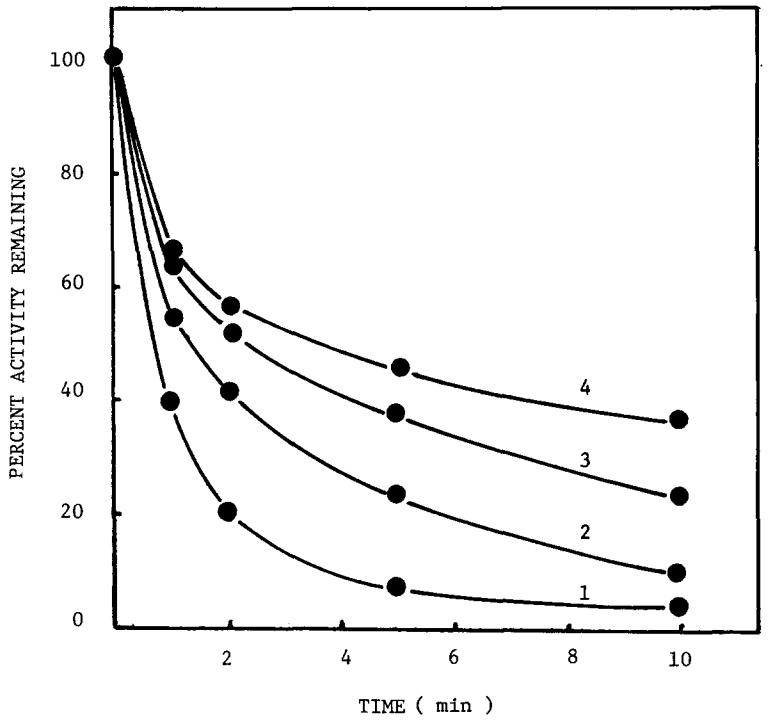

Fig. 2. Effect of Pyr-Val on inactivation of pyroglutamyl aminopeptidase by PGCK. The reaction mixtures consisted of 6 nM enzyme/7.7 nM PGCK/0.12 mM 2-mercaptoethanol with or without Pyr-Val in 0.1 M phosphate buffer, pH 6.0. The concentrations of Pyr-Val were: 1, none; 2, 0.1 mM; 3, 0.19 mM; 4, 0.58 mM. Aliquots of the reaction mixture were periodically removed and diluted 10-fold with 0.1 M phosphate buffer, pH 6.0. The residual activities were assayed by the fluorimetric method using Pyr-2-NNap as a substrate and expressed as the relative values against the control experiment in which no PGCK was added.

As illustrated in Fig. 1, when PGCK is incubated with the enzyme at concentration less than apparent molar equivalence to enzyme protein, inhibition is nearly complete in 10 min. This discrepancy is presumably due to the presence of catalytically inactive protein. Thus, the titration of pyroglutamyl aminopeptidase activity by PGCK was carried out in order to determine the concentration of the active enzyme from the stoichiometry of the enzyme inactivation. One of the electrophoretically homogeneous preparations was incubated with various concentrations of PGCK at 25° C for 2 h, and the remaining activities were determined. The linear relationship was observed between the concentration of PGCK and the degree of the enzyme inhibition, and the active enzyme concentration by this titration was calculated to be 61% of the total protein estimated by the method of Lowry et al. [20].

Discussion

The inhibition of pyroglutamyl aminopeptidase by PGCK occurs rapidly at a much lower concentration than chemically more reactive iodoacetate and other thiol group-blocking reagents. This rapid inactivation, as compared with

those by tosyl-lysyl chloromethyl ketone and tosyl phenylalanyl chloromethyl ketone for trypsin [22] and α-chymotrypsin [23], respectively, is consistant with greater nucleophilicity of a thiol group over an imidazole group, suggesting a cysteine residue as the site of alkylation in pyroglutamyl aminopeptidase, as in case of the other thiol proteases [13—16]. This is also supported by the fact that PCMB-inactivated enzyme did not react with PGCK and restore its activity by subsequent treatment with 2-mercaptoethanol. Moreover, the fact that a substrate, Pyr-Val, is very effective in protecting the enzyme from the reaction with PGCK, at concentrations greater than the K_m value, leaves little doubt in concluding that the reaction occurs with a group in the active site of the enzyme.

PGCK is a specific inhibitor for pyroglutamyl aminopeptidase, but not for papain, trypsin and α-chymotrypsin. This is entirely in accord with the substrate specificity of pyroglutamyl aminopeptidase. On the basis of the results described above, we could successfully use PGCK as a titrant to determine the normality of the active enzyme. The knowledge of the actual molar concentration of the active enzyme makes it possible to accurately determine kinetic parameters for pyroglutamyl aminopeptidase-catalyzed hydrolysis of various substrates.

Pyroglutamyl aminopeptidase is known to be an ubiquitous enzyme [1—10]. Thus, we also examined the reactivity of PGCK with pyroglutamyl aminopeptidases from rat liver and kidney using their tissue homogenates prepared freshly in the presence of 0.1 mM 2-mercaptoethanol. The enzyme activity in both homogenates hydrolyzing Pyr-2-NNap at a rate of 24 nmol/min, at 30°C, was inhibited completely within 10 min by 0.1 μM PGCK. These results suggest that PGCK might also act as an affinity labelling reagent for the enzyme in rat liver and kidney as expected.

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