A POSSIBLE ROLE FOR A SINGLE CYSTEINE RESIDUE IN CARBOXYPEPTIDASE Y

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1. Introduction

Carboxypeptidase Y obtained from bakers' yeast has been characterized as an enzyme of broad specificity [1]. The enzyme contains no metals [2] and is stoichiometrically inhibited by DFP** [2,3] and Z-PheCH_2Cl [4]. The reaction sites of DFP and Z-Phe-CH_2Cl have been shown to be serine and histidine residues, respectively. Thus, carboxypeptidase Y is a serine enzyme like chymotrypsin. However, carboxypeptidase Y is stoichiometrically inhibited by *p*-HMB. Chemical analyses of the enzyme revealed the presence of a single cysteine residue [3]. The *p*-HMB inhibited enzyme is not reactive with DFP [3]. Conversely, the DFP-inhibited enzyme is not reactive with *p*-HMB (to be published). Under these circumstances, there is the question as to how the SH-group of the enzyme functions.

The effect of aromatic mercurials on enzymatic activities toward various synthetic substrates was studied. It was found that the mercurial-treated enzyme retained activity toward the poor substrates [5], Z-Gly-Phe and Bz-Gly-L, β-phenyllactate, but lost activity toward the good substrates, Z-Phe-Leu and Ac-Phe-OEt. This led to the conclusion that the single cysteine residue of carboxypeptidase Y is not functionally essential but that it is situated at or near the substrate binding site.

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** *Abbreviations:* Ac, acetyl; Bz, benzoyl; DFP, diisopropylphosphofluoridate; OEt, ethylester; *p*-HMB, *p*-hydroxymercuribenzoate; *p*-HMS, *p*-hydroxymercuribenzene sulfonate; ONp, *p*-nitrophenyl ester; Z, benzyloxycarbonyl; Z-PheCH_2Cl, chloromethyl ketone derivative of Z-Phe. All amino acids had the L configuration, except glycine.

2. Materials and methods

Carboxypeptidase Y prepared form Fleischmann's yeast [1] was used. Z-Phe-Leu and Z-Phe-Gly were purchased from Fluka; Z-Gly-Phe from Calbiochem; *p*-nitrophenylacetate from Tokyo Kasei; *p*-nitrophenyltrimethylacetate from Aldrich; Ac-Phe-OEt from the Protein Research Foundation (Osaka); and Bz-Gly-L, β-phenyllactate from Seikagaku Kogyo. *p*-HMB was recrystallized. Other reagents were of analytical grade.

The assay for the N-substituted dipeptides was performed in 0.05 M of sodium phosphate, pH 6.5. Activity toward Z-Phe-Leu was spectrophotometrically measured [6] and other peptidase activity was determined by the ninhydrin method [5]. Activities toward Bz-Gly-L, β-phenyllactate and Ac-Phe-OEt were determined at pH 6.0 and pH 8.0, respectively, by the pH stat method [6]. The assays for *p*-nitrophenylacetate and *p*-nitrophenyltrimethylacetate were performed in 0.05 M sodium phosphate, pH 7.0, containing 2.5% *N,N*-dimethylformamide [7]. The activity was spectrophotometrically determined by measuring the release of *p*-nitrophenol at 400 nm. All assays were performed at 25°C.

3. Results and discussion

When carboxypeptidase Y was incubated with increasing amounts of aromatic mercurials, i.e. phenylmercuric chloride, *p*-aminophenylmercuric acetate, *p*-HMB and *p*-HMS, its activity toward Z-Phe-Leu was lost in parallel with the amount of the reagent. Total inactivation occurred at the equimolar quantities of the reagent and enzyme. The activity toward Ac-Phe-OEt was also completely lost by incubation with

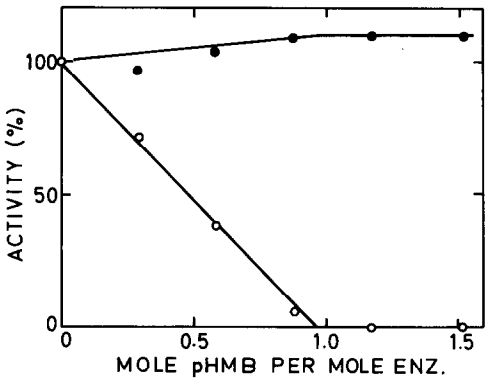


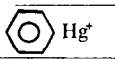
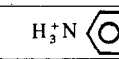
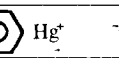
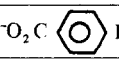
Fig.1. Effect of *p*-HMB on the carboxypeptidase Y-catalyzed hydrolysis of Z-Phe-Leu and Bz-Gly-L, β-phenyllactate. The enzyme was preincubated with increasing amounts of *p*-HMB in 0.083 M sodium phosphate, pH 7.0, at 20°C for 13 hr. After incubation, an aliquot of the mixture was used to determine the activity toward Z-Phe-Leu (-○-) and Bz-Gly-L, β-phenyllactate (-●-).

stoichiometric amount of *p*-HMB [3]. However, the activity toward Bz-Gly-L, β-phenyllactate remained unaffected or was slightly enhanced. An example with *p*-HMB treatment is shown in fig.1. The losses in activity caused by the mercurials were all recovered by incubation with 10^{-2} M cysteine.

The stoichiometric inactivation, as well as the reactivation by cysteine, strongly suggests that the mercurials used above form the mercaptide with the SH group of carboxypeptidase Y. Furthermore, using Boyer's titration method [8], we confirmed that *p*-HMB stoichiometrically reacted with the enzyme.

Carboxypeptidase Y was preincubated with aromatic mercurials and subjected to the hydrolysis of various synthetic substrates (table 1). The series of substrates was designed to determine the effect of the independent variation of the NH_2-blocking group (R_1), the penultimate amino acid (R_2) and the COOH-terminal group (R_3). The sensitive bond was between the R_2 and R_3. As shown in table 1, the activities of all the mercurial-treated enzymes toward Z-Phe-Leu were completely lost. Changing the R_1 from Z to Ac also resulted essentially in no activity. When the R_3 residue was glycine as in Z-Phe-Gly, the activity was also very small. However, when the R_2 residue is glycine, as in Z-Gly-Phe, the mercurial-treated enzymes retained their full or appreciable activities. This indicates that in the reaction with mercurials, the loss or gain in activity depends on the structure of the R_2 residue of the substrate. This is also true for the behavior of mercurial-treated enzymes toward *p*-nitrophenylacetate and trimethylacetate [7], in which the R_2 residue corresponds to the acetyl and trimethylacetyl groups, respectively: the mercurial-treated enzymes showed appreciable activity toward

Table 1
Activities of mercurial-treated carboxypeptidase Y toward various synthetic substrates

Substrate (R_1-R_2-R_3)	Treatment of the enzyme with			
				
	Activity relative to the native enzyme (%)			
Z-Phe-Leu	1	0	1	0
Ac-Phe-Leu	3	2	1	3
Z-Phe-Gly	7	9	5	0
Z-Gly-Phe	169	100	41	10
Acetyl-ONp[a]	81	41	52	44
Trimethylacetyl-ONp[b]	0	0	0	0

[a] *p*-Nitrophenylacetate.
[b] *p*-Nitrophenyltrimethylacetate.
The enzyme was incubated with a 1.2 molar excess of aromatic mercurials in 0.083 M of sodium phosphate, pH 7.0, at 20°C for 60 min. After incubation, an aliquot was used for the activity assay. Except for *p*-nitrophenylacetate, the substrate concentration was nearly equal or higher by several fold than the K_M values [5] of the native enzyme toward the respective substrate. The concentration of *p*-nitrophenylacetate was one half the K_M value [7].

p-nitrophenylacetate, but completely lost activity toward *p*-nitrophenyltrimethylacetate (table 1).

This phenomenon can be explained as being due to the fact that the mercurial derivatives of the enzyme retain activity toward substrates having a small R_2 residue, e.g. glycyl and acetyl groups, while they have no activity toward bulky R_2 residues. Thus, we conclude that the single cysteine residue of carboxypeptidase Y is not functionally essential. The SH group is believed to be situated near or at the subsite with which the R_2 residue of the substrate interacts; hence the reaction with the bulky mercurials leads to an inhibition of a substrate having the bulky R_2 residue, due to interference with the substrate binding, whereas activity is retained toward a substrate having a small R_2 residue. Further studies on the kinetic behavior of the mercurial enzymes are underway to confirm this hypothesis.

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