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FOR THE RECORD

Identification of the catalytic histidine residue participating in the charge-relay system of carboxypeptidase Y

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Abstract: The essential histidine residue of carboxypeptidase Y (CPY) was modified by a site-specific reagent, a chloromethylketone derivative of benzyloxycarbonyl-L-phenylalanine. The single modified histidine residue was converted to N^{τ}-carboxymethyl histidine (cmHis) upon performic acid oxidation. A peptide containing cmHis was isolated from the tryptic–thermolytic digest. Based on the amino acid composition and sequence analysis, the peptide is shown to be Val-Phe-Asp-Gly-Gly-cmHis-$MetO_2$-Val-Pro, which was derived from CPY cleaved by trypsin at Arg 391 and thermolysin at Phe 401, and thus His 397 was modified. This histidine residue has been implicated previously by X-ray analysis to participate in the charge-relay system of CPY.

Keywords: active site; benzyloxycarbonyl-L-phenylalanine chloromethylketone; carboxymethyl histidine; carboxypeptidase Y; charge-relay system; chemical modification

Carboxypeptidase Y from baker's yeast belongs to the group of serine carboxypeptidases which, like serine endopeptidases, have a charge-relay system at the active center (Hayashi et al., 1975a; Kuhn et al., 1976; Bech & Breddam, 1989). The charge-relay system consists of three amino acid residues in which the serine residue is linked through the imidazole ring of the histidine to the carboxylate anion of the aspartic acid. The combination of these three amino acid residues produces the enhancing nucleophilicity of the serine hydroxyl (Blow et al., 1969). For CPY, the essential serine residue is Ser 146, as identified by chemical modification (Hayashi et al., 1973b, 1975b). Chloromethylketone derivatives, which have been widely used to specifically modify the essential histidyl residue in many serine endopeptidases (Shaw, 1967), have also been shown to react with a single histidine residue in CPY (Hayashi et al., 1975a). Although His 397 was implicated to be the essential residue by X-ray study (Endrizzi et al., 1994), the exact position of this essential histidine residue in CPY was not identified by the chemical modification study with chloromethylketone derivatives (Hayashi et al., 1975a). Herein, we report the identification of the position of the modified histidine residue.

A preparation of CPY, obtained from Oriental Yeast Co. (lot OY 73-11), was prepared according to the method described previously (Hayashi et al., 1975a), and CPY in this preparation was further purified by hydroxylapatite column chromatography essentially according to the procedure of Bernardi (1971). At 4 °C, the preparation was loaded onto a hydroxylapatite column equilibrated with 75 mM sodium phosphate buffer, pH 6.8, and CPY was eluted with 150 mM buffer. The enzyme solution was desalted on an ultrafiltration apparatus (Amicon Stirred Cells 8050) by repeated concentration and dilution with water and finally concentrated to a protein concentration of 2 mg/mL. Z-Phe-CH_2Cl was synthesized as described previously (Hayashi et al., 1975a). For assays of the CPY activity, the spectrophotometric method of Hayashi (1976) was adopted, except that aliquots of 2 μL of sample solution were pipetted into the pH 7 assay buffer containing 3 mM benzoyl-L-tyrosine *p*-nitroanilide. The enzyme molar concentration was calculated based on absorbance at 280 nm of 15.0 for 1% protein solution and a molecular weight of 61,000 (Aibara et al., 1971; Hayashi et al., 1973a). For evaporation of solvents, a Savant VaporNet AES 1000 Speedvac concentrator was used. To convert the chloromethylketone-reacted His to cmHis, the inactivated CPY was treated with performic acid at 0 °C for 2.5 h according to the performic acid oxidation method of Liao et al. (1973). The oxidation reaction was stopped by evaporation of the sample to dryness. Approximately 15 mg of CPY was incubated at 25 °C with Z-Phe-CH_2Cl at a 20-fold molar excess of the reagent to protein in 10 mM sodium phosphate buffer, pH 6.0, as described

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Abbreviations: CPY, carboxypeptidase Y; cmHis, N^{τ}-carboxymethyl histidine; $MetO_2$, methionine sulfone; PTH, phenylthiohydantoin; TFA, trifluoracetic acid; Z-Phe-CH_2Cl, benzyloxycarbonyl-L-phenylalanine chloromethylketone.

Table 1. *Amino acid composition of the cmHis-containing peptide hydrolyzed for 24 h (Hirs, 1956)*

Residue[a]	Observed (residues/mol)	Theoretical[b] (residues/mol)
Asp	1.2	1
Pro	1.0	1
Gly	2.0	2
Val	1.6	2
Phe	0.8	1
cmHis[c]	1.2	1
$MetO_2$	1.0	1

[a] Amino acids with less than 0.2 residues/mol are not listed.
[b] Based upon a tryptic-thermolytic peptide sequence of CPY (residues 392–340).
[c] Amino acid analyses were performed with a JEOL JLC-300 amino acid analyzer. Both Ala and cmHis were eluted at 14 min on the amino acid analyzer with the regular program. This 14-min peak was later identified as cmHis with a modified program in which cmHis appeared as a single peak between Ala and 1/2 Cys. In the modified program, the analytical column temperature was reduced to 35 °C from 45 °C during the first buffer (pH 3.15) elution. The color value of Ala was used for that of cmHis. A mixture of N^{π}-, N^{τ}-, and $N^{\pi,\tau}$-cmHis was prepared according to Crestfield et al. (1963) and was used without further purification to identify N^{τ}-cmHis on the amino acid analyzer.

Table 2. *Yields of PTH-amino acids obtained from the protein sequencer*[a]

Cycle	PTH-amino acid	Amount found (pmol)
1	Val	930
2	Phe	613
3	Asp	25
4	Gly	102
5	Gly	115
6	None	0
7	$MetO_2$	46
8	Val	95
9	Pro	25

[a] An Applied Biosystems model 476 A protein sequencer was used for the peptide sequencing.

previously (Hayashi et al., 1975a). The inhibited enzyme solution was filtered through glass wool to remove the insoluble Z-Phe-CH_2Cl and evaporated to dryness under vacuum prior to performic acid oxidation. For the tryptic digestion, the inactivated CPY sample was dissolved in 1% ammonium bicarbonate, to which were added two aliquots (2% w/v) of tosyl phenylalanine chloromethylketone-treated trypsin (Cooper Biomedical, Washington, D.C.) with an incubation time of 2 h for each addition and temperature maintained at 37 °C (Greene & Bartelt, 1977). The trypsin-digested CPY peptides were fractionated on a Sephadex G-50 column (0.9 × 60 cm). The eluent fractions having cmHis-containing peptides were further digested with thermolysin (Nacalai Tesque, Inc., Kyoto, Japan). The digestion was for 4 h at 45 °C with thermolysin at about 2% of total peptide amount by weight (total peptide amount was based on absorbance at 280 nm of the pooled fractions). The thermolysin-digested peptides were fractionated on a Sephadex G-25 column (0.8 × 50 cm). All of the above gel chromatography columns were equilibrated and eluted with 1% ammonium bicarbonate. The identification of cmHis-containing peptides was performed on an amino acid analyzer after acid hydrolysis (Moore & Stein, 1963). The amino acid, cmHis, was eluted as a single peak on the amino acid analyzer with a modified program (see footnote c of Table 1). The single cmHis-containing peptide in the Sephadex G-25 fractions was purified by HPLC as shown in Figure 1. The purified cmHis-containing peptide consists of nine amino acid residues (Table 1), and the sequence of the peptide was elucidated as Val-Phe-Asp-Gly-Gly-cmHis-$MetO_2$-Val-Pro (Table 2). Although PTH-cmHis was not identified in cycle 6, the amino acid composition (Table 1) indeed indicated the presence of cmHis. This sequence was aligned with a peptide from Val 392 to Pro 400 (Val-Phe-Asn-Gly-Gly-His-Met-Val-Pro) in the primary structure of CPY (Breddam & Svendsen, 1984). The result indicates that His 397 was alkylated by Z-Phe-CH_2Cl and converted to cmHis by performic acid, which concomitantly oxidized Met 398 to methionine sulfone. The sequencing analyses showing only PTH-Asp and none of PTH-Asn in cycle 3, with a sudden yield drop (Table 2), may be attributed to the Asn-Gly peptide bond cyclization. It is known that the Asn-Gly peptide bond can form cyclic imide under acidic conditions and give rise to the α- and β-Asp-Gly peptide bonds under alkali conditions (DeTar et al., 1967a, 1967b). The cyclized product as well as the β-Asp-Gly peptide bond may thus stop the Edman degradation reaction in the subsequent cycles.

On the basis of homology among five different serine carboxypeptidases, the conserved His 397 was suggested as the essential histidine residue of CPY (Bech & Breddam, 1989). The X-ray crystallographic study has also shown that His 397 locates near Ser 146, the other active site residue (Endrizzi et al., 1994).

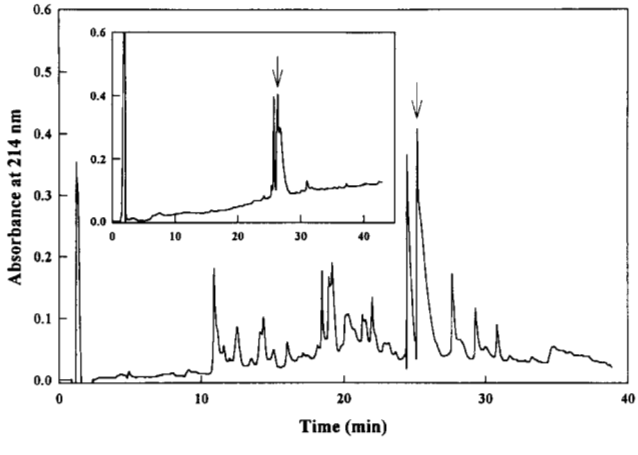

Fig. 1. HPLC profile for purification of the cmHis-containing peptide. HPLC for peptide fractionation was performed on a Shimadzu LC-10AS HPLC system with a Waters NOVA-PAK ODS (4.6 × 150 mm) column. The initial solvent was 0.1% TFA in H_2O, and the limiting solvent was 0.085% TFA in 90% aqueous acetonitrile. Peptides were eluted by a linear gradient with the limiting solvent set at 47% in 60 min. **Inset:** For final purification of the cmHis-containing peptide, the concentration of TFA in the initial and limiting solvents was decreased to 0.01%. The arrow indicates the peak whose eluent contains cmHis in the hydrolysate.

Also suggested was Glu 145, which may interact with His 397 via carboxylic group substrates and may indirectly be involved in the catalytic role (Christensen, 1994). Here, we show that the essential histidine residue of CPY modified by Z-Phe-CH_2Cl is His 397. This is the first serine carboxypeptidase whose essential histidine residue has been identified by chemical alkylation. Whether other serine carboxypeptidases will behave similarly as does CPY would be of interest for further investigation.

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