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Proton-Relay System of Carboxypeptidase Y as a Sole Catalytic Site: Studies on Mutagenic Replacement of His 397

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His397 was replaced with alanine by site-directed mutagenesis of the cloned PRC1 gene in order to confirm the role of this residue in the proton-relay system of carboxypeptidase Y (CPY). The expressed and purified H397A showed a CD spectrum almost identical to that of the wild type enzyme, but its heat stability and conformation on heating differed somewhat. Kinetic analysis showed that the k_{cat} values of the purified H397A toward the peptide substrates, Z-Phe-Leu and Z-Gly-Phe, were reduced to approximately 4×10^{-5}-fold, whereas the K_m values remained almost unchanged. The activity of the H397A preparation with the ester substrate, Ac-Phe-OEt, was negligible. The low activity of our H397A was lost on treatment with DFP and Z-Phe-CH_2Cl, site-specific inhibitors, respectively, for Ser146 and His397, and with the $HgCl_2$ and PCMB, SH-reagents for Cys341. After treatment with these inhibitors, the k_{cat} value for the H397A preparation toward Z-Phe-Leu decreased 1×10^3-fold or more. The value was approximately 10^{-8} for the wild type enzyme. This level of activity is 10^3-fold lower than the reported value for the same mutant of CPY [*Carlsberg Res. Commun.* 54, 165-171 (1989)], and more than 10-fold lower than the values for the corresponding His-to-Ala mutants of trypsin [*J. Am. Chem. Soc.* 114, 1784-1790 (1992)] and subtilisin [*Nature* 332, 564-568 (1988)]. These findings, together with the pH profiles and chromatographic behavior, are evidence that the low activity of the H397A preparation is due to contamination by wild type CPY. The decreased k_{cat} value of our H397A mutant is the lowest reported among the corresponding histidine mutants of serine proteases. We conclude that the proton-relay system composed of Ser146 and His397 is the sole catalytic center of CPY, and that its destruction leads to complete inactivation.

Key words: carboxypeptidase Y, catalytic histidine, proton-relay system, site-directed mutagenesis

Carboxypeptidase Y (CPY) from bakers' yeast belongs to a group of serine carboxypeptidases which, like the serine endopeptidases, have a catalytic triad at their active centers (*1, 2*), because Ser146 and His397 have been identified as residues important for enzyme activity by chemical modification (*3-5*). Mutagenic replacement of these two residues has shown their importance in catalytic functions (*6, 7*). Mutant enzymes, however, in particular H397A and H397R, exhibit higher activity than similar histidine mutants of serine endopeptidases, trypsin and subtilisin (*8, 9*).

The unexpectedly high activities of the histidine mutants of CPY raises questions as to whether or not the structure of the catalytic triad in CPY differs from that in other serine endopeptidases, and whether or not the catalytic triad is the sole catalytic center of CPY (for example, see Bech and Breddam, 1989). These questions are complicated by different evaluations of the role of the single SH group, Cys341, in CPY. Cys341 is located in the substrate binding site (*10*) but has a function which is very sensitive to modification (*11*). The high hydrolytic activity of CPY shown toward peptide substrates in a low pH range (*7, 12-14*) also raises a question as to the catalytic triad of this enzyme. Recently, Stennicke *et al.* mentioned contamination by an unidentified serine-type carboxypeptidase of a mutant enzyme preparation (*7*). To answer these questions, we thoroughly investigated the properties of the histidine mutant enzyme, H397A.

MATERIALS AND METHODS

Materials—Plasmid pTSY3, bearing the PRC1 gene coding for CPY, was a kind gift from Dr. Klaus Breddam, the Carlsberg Laboratory, Copenhagen, Denmark. The synthetic oligonucleotides were obtained from Japan Bio Services. The restriction endonucleases and T4 polynucleotide kinase were purchased from Toyobo. The transformer™ site-directed mutagenesis kit was bought from Clontech, and the Taq dyedeoxy™ terminator cycle sequencing kit from Applied Biosystems.

Wild type CPY (Lot OY 73-11) was obtained from

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Abbreviations: BSA, bovine serum albumin; CPY, carboxypeptidase Y; CD, circular dichroism; CNBr, cyanogen bromide; DFP, diisopropylphosphorofluoridate; -OEt, ethylester; PCMB, *p*-chloromercuribenzoic acid; PMSF, phenylmethylsulfonylfluoride; Z, benzyloxycarbonyl; Z-Phe-CH_2Cl, chloromethylketone derivative of Z-Phe.

Oriental Yeast. Z-PheCH$_2$Cl was synthesized as described previously (*1*) or was purchased from Fox Chemical. DFP and [^{3}H]DFP, respectively, were obtained from Wako and Du Pont NEN Research Laboratories. Bis-Tris, $HgCl_2$, and phenylmercuric acetate were purchased from Nacalai Tesque. DEAE-Sephadex A-50 was bought from Pharmacia Fine Chemicals. Hydroxylapatite gel was purchased from Bio-Rad. Z-Phe-Leu was obtained from Sigma Chemical Company, and Z-Gly-Phe from Calbiochem. All the other chemicals and solvents used were of analytical grade.

Chemical Analysis—Amino acid analysis was performed with an amino acid analyzer (JEOL JLC-300). pH-stat measurements were made with a Radiometer, model PHM290. HPLC was performed with a Shimadzu LC-10AS HPLC system equipped with a Waters NOVA-PAK ODS (4.6×150 mm) column. The N-terminal amino acid sequence was determined in a protein sequencer (Applied Biosystems model 476 A). An ultrafiltration system in a PM 30 membrane (Amicon) was used. A concentrator (Savant VaporNet AES 1000 Speedvac) was used to evaporate the solvents. DNA sequencing was performed with an Applied Biosystems 373A DNA Sequencer and by PCR with a Perkin Elmer Cetus model 480. UV-VIS spectra were measured with a spectrophotometer (Shimadzu UV-160A or UV-2500PC spectrophotometer). CD were measured with a spectropolarimeter at room temperature (JASCO J-720W). Six scans were averaged for the wild type and H397A CPY at respective concentrations of 2.3 and 3.3 μM. A Hoefer Mighty Small single slab unit was used for SDS-PAGE. Incorporation of [^{3}H]DFP was measured with a Packard 1600TR scintillation counter.

Site-Directed Mutagenesis—*In vitro* mutagenesis was performed with the pTSY3 subclone of the PRC1 gene (*6*). Three oligonucleotides were used for mutation: a 29 mer as the mutagenic primer to introduce an alanyl residue for His397, sequence 5′>C TTC AAT GGT GGC GCC ATG GTT CCA TTT G<3′; a 26 mer as the selection primer, 5′>A CTA CAA AAT GAG CTC CCT CGC GCGT<3′, to introduce a *Pvu*II restriction site into plasmid pTSY3 for selection; and a 20 mer sequence primer for DNA sequencing, 5′>G CCA TGG AAG TAC GAC GAA G<3′. Mutation was performed with a Transformer™ site-directed mutagenesis kit (Clontech) as described by Deng and Nickoloff (*15*). The DNA sequence reaction was performed with a Taq dyedeoxy™ terminator cycle sequencing kit. Yeast strain SEY2202 was transformed by the lithium acetate method (*16*). *Escherichia coli* JM109 was transformed by the method of Hanahan using a standard transformation buffer (*17*).

Strains—*E. coli* BMH71-18mutS (*thi supE Δ(lac-proAB) [mutS::Tn10] F′[proAB$^+$ laqI^q lacZΔM15]*) and *E. coli* JM 109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB) F′[traD36 proAB$^+$ laqI^q lacZΔ M15]*) were used for plasmid propagation. *Saccharomyces cerevisiae* SEY2202 (*MATa ΔprcI:: (LEU2) leu2-3, 112 ura3-52 his4-519*) was used as the host for transformation.

Activity Determination—Peptidase activity was determined with 0.4 mM Z-Phe-Leu in 0.05 M bis-Tris buffer containing 1 mM EDTA, pH 6.5, at 25°C (*18*). The amounts of leucine released were determined with an amino acid analyzer. Esterase activity toward Ac-Phe-OEt was examined by three methods: (i) Spectrophotometry was performed according to the procedure of Hayashi (*18*). (ii) The pH-stat technique was performed under a nitrogen stream at 25°C (*12*). (iii) The reaction product, Ac-Phe, was detected by HPLC and monitored as to the absorbance of 260 nm. All the esterase activities were determined at pH 7.5.

Enzyme Purification—The wild type and H397A CPY, prepared as described previously (*4, 19*), were purified by hydroxylapatite column chromatography (*20*): The column was equilibrated with 75 mM sodium phosphate buffer, pH 6.8, after which CPY was eluted with 150 mM sodium phosphate buffer, pH 6.8. All chromatographic procedures were performed at 4°C. The salts were replaced with water by ultrafiltration. The aqueous protein solution, concentrated to 2 mg/ml, was used as the standard preparation throughout the experiment. The purity of the enzyme was determined by SDS-PAGE and N-terminal amino acid sequencing. Second hydroxylapatite chromatography was performed to check the purity of the H397A preparation. For this chromatography, the column was washed with 90 mM sodium phosphate buffer, pH 6.8, and the protein was eluted with 130 mM sodium phosphate buffer.

Characterization of the Wild Type and H397A CPY—The molar concentration was calculated taking the absorbance at 280 nm of a 1% protein solution as 15.0 and the molecular weights 61,000 (*19, 21*). Enzymatic hydrolysis of Z-dipeptides was performed at 25°C in 0.05 M bis-Tris buffer containing 1 mM EDTA, pH 6.5. The amounts of amino acids released were determined with an amino acid analyzer (*18*). Kinetic parameters, *i.e.* the k_{cat} and K_m values, were calculated from Lineweaver-Burk plots.

Reaction with Site-Specific Reagents—To determine the inhibitory effect of DFP on peptidase activity, the wild type and H397A CPY were incubated with a 1/200-fold molar excess of DFP. The protein concentration was maintained at 20 μM by adding BSA (usually a 2.7×10^4-fold molar excess) when an extremely small amount of the wild type enzyme was used. Enzyme inhibition by Z-PheCH$_2$Cl was assayed with a 20-fold molar excess of the reagent as to the protein. Residual enzyme activity was monitored during the incubation.

In the DFP incorporation experiments, a 1.5-fold molar excess of [^{3}H]DFP was mixed with each enzyme, and the whole mixture was incubated in 0.05 M bis-Tris buffer containing 1 mM EDTA, pH 6.5, for 1 h at 25°C. Excess reagents were removed by ultrafiltration, and the radioactivity incorporated in the enzyme was measured for 10 min with a liquid scintillation counter. Reactions with the SH reagents, $HgCl_2$ and PCMB, were performed for 1 min at 25°C with a 2-fold molar excess of each inhibitor.

RESULTS

Structural Properties of H397A—The yields of the wild type and H397A CPY changed in parallel during purification (Table I). The chromatographic behavior of H397A was very similar to that of the wild type CPY (*18*). After the first hydroxylapatite chromatography the purified enzymes gave single bands on SDS-PAGE and a single N-terminal amino acid, lysine, on sequencing. The CD spectra of the wild type and H397A CPY in the far-UV region were almost identical (Fig. 1). These results show that H397A was correctly expressed in the host cells and

was folded, forming the same gross structure as the wild type enzyme.

Several differences, however, exist between the wild type and mutant CPY. Their heat stabilities differ, the denaturation temperatures, Tm, of the respective enzymes being 55 and 60°C (Fig. 2). In addition, they have different secondary structures after heat treatment. These findings are evidence of changes in the stability and structural flexibility of H397A. Furthermore, when the protein, obtained as a single peak in the second hydroxylapatite chromatography, was divided into two fractions, the specific activity of the H397A preparation in the pooled front half was 10-fold that in the pooled back half.

Enzymatic Properties of H397A—The kinetic constants of the wild type and H397A CPY toward Z-Phe-Leu and Z-Gly-Phe are shown in Table II. The H397A k_{cat} values for both substrates were smaller by approximately 4×10^{-5} than those of the wild type CPY, whereas the K_m values of both enzymes were nearly the same for each substrate. As to esterase activity toward Ac-Phe-OEt, the wild type CPY showed normal activity, whereas the H397A preparation showed negligible or no activity under the experimental conditions used.

Both the wild type and H397A CPY exhibited nearly

TABLE I. **Wild type and H397A CPY yields on purification from 100 g of yeast cells.**

Purification step[a]	Wild type CPY		H397A CPY
	Total protein (mg)	Total activity[b] (units)	Total protein (mg)
After activation treatment	619.8	10.6	569.6
Stepwise chromatography on DEAE Sephadex A-50	46.4	8.5	32.2
Linear gradient chromatography on DEAE Sephadex A-50	4.95	4.5	5.05
Chromatography on hydroxylapatite	3.13	2.6	3.41

[a]Enzymes were purified by the method of Hayashi *et al.* (*19*). [b]Activity toward Bz-Tyr-pNA was determined (*18*).

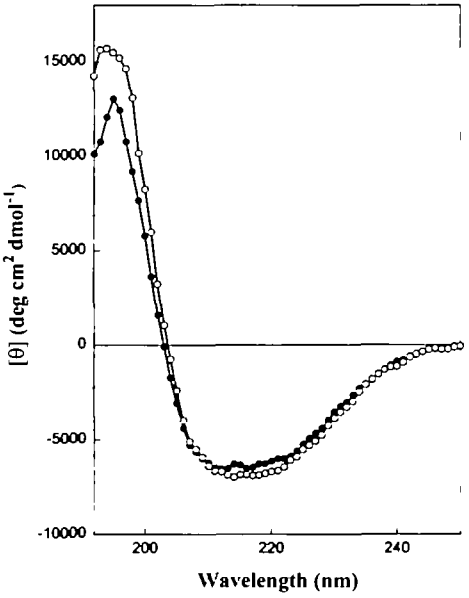

Fig. 1. **CD spectra of the wild type and H397A CPY.** The protein concentrations of CPY and its mutant were, respectively, 2.3 and 3.3 μM. Measurement conditions: band width, 1.0 nm; sensitivity, 50 mdeg; response, 0.5 s; wave length, 190-250 nm; scan speed, 100 nm/min; step resolution, 1 nm; six measurements being made. ●, wild type CPY; ○, H397A.

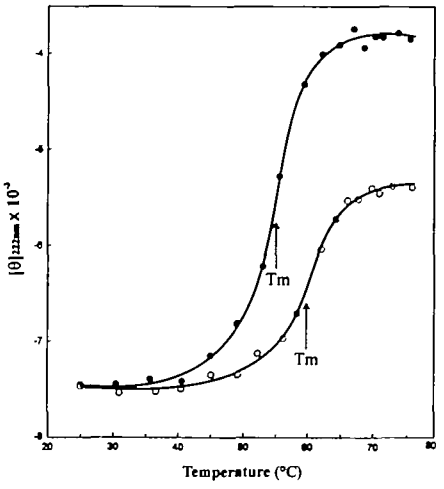

Fig. 2. **Thermal stability of the wild type and H397A CPY at CD 222 nm.** The temperature was increased at the rate of 0.65°C/min, and 1.1×10^{-3} mM of each enzyme was used. See Fig. 1 for other details. ●, wild type CPY; ○, H397A.

TABLE II. **Kinetic parameters of the wild type and H397A CPY as to the hydrolysis of Z-Gly-Phe and Z-Phe-Leu.**

Substrate	Enzyme	k_{cat} (min^{-1})	k_{cat} (ratio)	K_m (mM)	k_{cat}/K_m (min^{-1}·mM^{-1})
Z-Gly-Phe	CPY	110	1	0.50	220
	H397A	5.10×10^{-3}	4.6×10^{-5}	0.49	1.04×10^{-2}
Z-Phe-Leu	CPY	2,900	1	0.036	81,000
	H397A	1.07×10^{-1}	3.7×10^{-5}	0.042	2.64

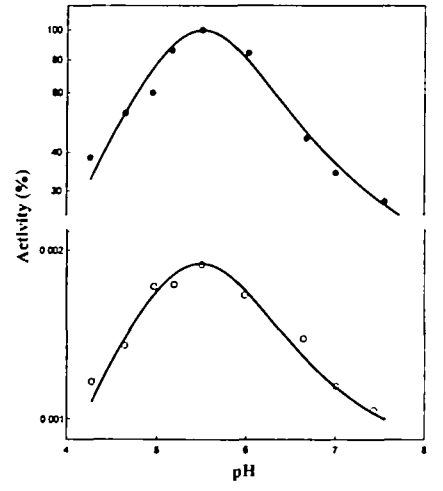

Fig. 3. **pH dependency of the wild type and H397A CPY toward Z-Phe-Leu.** The enzymatic assay was performed with an amino acid analyzer at 25°C with 0.4 mM Z-Phe-Leu, the release of leucine being measured. 0.05 M buffers (sodium acetate for pH 4.2-6.0, bis-Tris for pH 6.5 and 7.0, and HEPES for pH 7.5) were used. The activity of the wild type CPY at pH 5.5 was assumed to be 100%. ●, wild type CPY; ○, H397A.

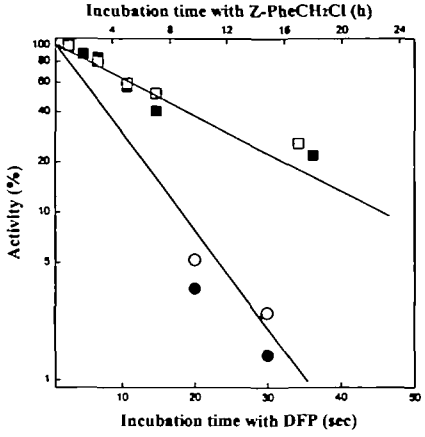

Fig. 4. **Inhibition of the wild type and H397A CPY by DFP and Z-PheCH$_2$Cl.** The assay mixture for the wild type CPY contained 7.5×10^{-7} mM CPY, 0.02 mM BSA, and 1×10^{-4} mM DFP. The assay conditions for H397A were the same, except that BSA was omitted. A 20-fold molar excess of Z-PheCH$_2$Cl was added to 3.6×10^{-4} mM wild type CPY and 0.02 mM H397A preparation. Peptidase activity toward 0.4 mM Z-Phe-Leu was determined at pH 6.5 and 25°C, by measuring the leucine released with an amino acid analyzer. ●, ○: DFP inhibition of the wild-type CPY and H397A, respectively. ■, □: Z-PheCH$_2$Cl inhibition of the wild type and H397A CPY, respectively.

identical pH profiles for peptidase activity towards Z-Phe-Leu (Fig. 3).

Reaction with Inhibitors—DFP inactivated the wild type and H397A CPY at the same rate, with first order reaction kinetics (Fig. 4). The pseudo-first order reaction rate, k_{1st}, for the inactivation was 0.14 s^{-1}. The extent of incorporation of [^{3}H]DFP into the H397A mutant was less than 10^4-fold compared to the value for the wild type enzyme (Table III). This level of ^{3}H incorporation was about the same as that for BSA, which we used as a control protein. After treatment with this inhibitor, the k_{cat} value for the H397A preparation toward Z-Phe-Leu decreased 1×10^3-fold or more. The value was approximately 10^{-8} for the wild type enzyme. Z-PheCH$_2$Cl also inactivated the H397A preparation at the same rate as it did the wild type CPY, and this inactivation followed first order reaction kinetics (Fig. 4), the k_{1st} for the inactivation being 2.6×10^{-5} s^{-1}. The SH reagents, $HgCl_2$ and PCMB, which react with a single cysteine residue, Cys 341 (*6, 10*), almost completely abolished the Z-Phe-Leu hydrolyzing activity of the wild type and H397A CPY.

DISCUSSION

Our H397A preparation exhibited fairly strong catalytic efficiency (k_{cat}/K_m); 10- to 100-fold those of the corresponding histidine mutants of trypsin and subtilisin (*8, 9*). Similar results have been reported for an H397A CPY preparation (*6*). These activities of histidine mutants of serine proteases have been ascribed to their intrinsic activity. In the case of the H397A CPY, this activity is presumed to be produced due to the participation of Cys341, located at the S_1 binding site (*6*).

Our findings indicate that the activity of the H397A preparation is due to contamination by the wild type CPY,

TABLE III. **Incorporation of [^{3}H]DFP into the wild type and H397A CPY.**[a]

Enzyme	Radioactivity	
	cpm[b]	Ratio
CPY	132,500±100	1
H397A	12±1.0	1.1×10^{-4}
BSA[c]	10±1.0	1.3×10^{-4}

[a]A 1.5 molar excess of [^{3}H]DFP (310.8 kBq/nmol) was added to 0.07 nmol of each sample protein for labeling. Each mixture was then incubated at 25°C for 1 h in 0.05 M bis-Tris containing 1 mM EDTA, pH 6.5. Free [^{3}H]DFP was removed by ultrafiltration before liquid scintillation counting. The cpm values are the averages for three experiments. [b]Counts per minute. [c]Bovine serum albumin was used in the control experiment.

and not to any intrinsic activity of H397A. Several lines of evidence support this view: (i) The K_m values towards two peptide substrates were almost the same for both H397A and the wild-type CPY (Table II). This was inconsistent with in the case of subtilisin, where different K_m values were found (*9*). Moreover, the k_{cat} ratio of the wild type and mutant enzymes for Z-Phe-Leu and that for Z-Gly-Phe are almost the same (Table II). (ii) Because the H397A mutant lacks a pH-sensitive histidine residue, it is not expected to exhibit the same pH-profile as the wild type CPY (*7, 14*); but, the pH peptidase activity profiles of the wild type and H397A CPY are identical (Fig. 3). (iii) DFP inactivates H397A as efficiently as the wild type CPY (Fig. 4). (iv) Z-PheCH$_2$Cl inactivates the wild type and H397A CPY mutant at almost the same rate (Fig. 4). Because Z-PheCH$_2$Cl irreversibly alkylates the N^r position of His 397 (*1, 5*), there is no reason for Z-PheCH$_2$Cl to bind to H397A. (v) The results of the second hydroxylapatite chromatography suggest the presence of the wild type enzyme even as an apparently single peak on chromatograms.

As to the reason why the wild type enzyme contaminated the H397A preparation, misreading of the mutant codon at the tRNA level and the subsequent insertion of histidine into the nascent polypeptide is one possibility (*8, 22*). In our investigation, however, the H397A mutation was expressed by replacing the histidine codon, CAC, with an alanine codon, GCC, to minimize codon level misincorporation. Another possibility is that the wild type CPY may be introduced through plasmid contamination by the wild type enzyme or through an unknown endogenous wild type enzyme present in the host cells, SEY 2202. However, the former possibility can be ruled out because repeated mutagenesis experiments gave the H397A enzyme with identical properties. Thus, the latter possibility seems to be probable for the contamination by the wild type CPY. This was further confirmed by the fact that the contaminating wild type CPY activity, which was inhibited by DFP and Z-PheCH$_2$Cl, was detected when the enzyme was prepared in the host cells, SEY 2202, in a blank study (data not shown). Whatever the reason, the activity of the H397A preparation has now been attributed to wild type contamination.

Under the present experimental conditions, it was impossible to remove the extremely small level of wild type contamination from our H397A preparation. Both DFP- and Z-PheCH$_2$Cl-treated H397A showed trace activity, that is, 10- to 500-fold lower than those of other known serine endopeptidase mutants (*8, 9*). The trace peptidase

activity showed no pH-dependency like that of the wild type CPY (data not shown). There is no doubt, therefore, that His397 is an essential residue for the catalytic function of CPY, and that it is the sole catalytic center linked with Ser146, as proposed earlier by Blow *et al.* (*23*).

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