Carboxypeptidase Y activity and maintenance is modulated by a large helical structure

Mai Makino¹, Takehiko Sahara², Naoki Morita³ and Hiroshi Ueno⁴

¹ Department of Biochemistry, Nara Medical University, Kashihara, Japan
² Bio-Design Research Group, Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan
³ Molecular and Biological Technology Research Group, Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Sapporo, Japan
⁴ Laboratory of Biochemistry and Applied Microbiology, School of Agriculture, Ryukoku University, Otsu, Japan

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Correspondence
M. Makino, Department of Biochemistry, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan
Fax: +81-744-24-9525
E-mail: m.makino@naramed-u.ac.jp

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Carboxypeptidase Y (CPY) is a vacuolar serine-type protease isolated from yeast, Saccharomyces cerevisiae. CPY undergoes several maturation steps to form mature CPY (mCPY) that exhibits exopeptidase activity toward peptides, esters, amides, and anilides with broad substrate specificity [1–4]. mCPY has been crystallized, and its three-dimensional structure was determined [5]. Analysis of the structural anatomy of CPY using computer graphics revealed that mCPY has a typical α/β hydrolase fold pattern with two domains: a core domain (1–179 and 318–421) and an ‘insertion’ domain (180–317; Fig. 1A). The topology of the core domain of CPY is similar to that of the canonical core of the α/β hydrolase fold family [6] (Fig. 1B). The residues that form the catalytic triad, S146, H397, and D338, are all located within the core domain. The insertion domain contains helices that are attached to the conserved core folding topology (Fig. 1B). This helical insertion topology is often found in the α/β hydrolase fold family. Each insertion may have unique roles: For example, an insertion domain acts as a helical lid in most lipases where it helps control the access of substrate to the active site by blocking the entry of solvent to shield the active site pocket [7,8]; three insertions in acetylcholinesterase are proposed to be involved in channeling the substrates to the active site [9,10].

As a part of the insertion domain, CPY has two adjacent large α-helices, collectively termed the ‘V-shape helix’. Examination of the crystal structure of mCPY has revealed that the V-shape helix juts out from the core domain, reminiscent of a bird wing. The V-shape helix appears to cover the active site cavity. Structurally, the V-shape helix features four

Abbreviations
CPWII, wheat carboxypeptidase II; CPY, carboxypeptidase Y; ERAD, endoplasmic reticulum-associated degradation; mCPY, mature CPY; PPCA, protective protein/cathepsin A; pro-CPY, precursor CPY.
intramolecular isulfide bonds (Fig. 1C). Two disulfide bonds are located near the beginning (C193–C207) and the end (C262–C268) of the V-shape helix. The two other disulfide bonds, C217–C240 and C224–C233, bridge two α-helices that form the V-shape helix [11]. The absence of hydrogen bonds between the V-shape helix and the core domain supports the view that the V-shape helix plays a role as a flexible gate for the entry of substrate into the binding pocket. A similar V-shape helix is present in homologous serine carboxypeptidases, including wheat carboxypeptidase II (CPWII) and human protective protein/cathepsin A (PPCA), although the sequence similarity is low [12,13]. Based on these considerations, it is reasonable to suggest that the V-shape helix is a common motif among serine carboxypeptidases that contain an α/β hydrolase fold and that the helix plays significant role(s) in catalytic activities.

In this study, we investigated whether or not the V-shape helix is involved in the activity of CPY by constructing mutant CPYs in which the C193–C207 or C262–C268 disulfide bonds present in the V-shape helix were disrupted using site-directed mutagenesis.

Materials and methods

Chemicals

Blend Taq® and Taq DNA polymerase were purchased from TOYOBO (Osaka, Japan) and New England Biolabs (Ipswich, MA, USA), respectively. T4 DNA Ligase was purchased from Takara Bio (Kusatsu, Japan). Restriction enzymes were purchased from Takara Bio and TOYOBO.
Other general reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan).

**Protein manipulation**

Visualization of molecular structures was carried out using the Accelrys Discovery Studio Visualizer 4.0 (BIOVIA, Dassault Systèmes, San Diego, CA, USA). Atomic coordinate data—1YSC, 3SC2, 1IVY, and 4MWS—were obtained from the RCSB Protein Data Bank (http://www.rcsb.org) for mCPY, CPWII, precursor PPCA, and mature PPCA, respectively.

**Plasmid construction**

Sequence data of CPY, pre1/YMR297W (SGD ID: SGD: S000004912), were obtained from the Saccharomyces Genome Database (http://www.yeastgenome.org/). The recombinant wild-type CPY expression vector was prepared as described [14]: The pre1 fragment encoding full-length wild-type CPY was prepared from yeast genomic DNA extracted from yeast wild-type strain using PCR amplification with primers containing a SmaI or XhoI restriction site for the 5'- and 3'-termini, respectively. This fragment was inserted into the pGEM®-T Easy Vector (Promega, Madison, WI, USA) to construct the cloning vector, pLTex321sV5H[15], was digested with SmaI and XhoI to generate an insert fragment. Separately, the yeast expression vector, pLTrx432sV5H [15], was digested with SmaI and XhoI to generate the template fragment. The two fragments were ligated to construct the recombinant wild-type CPY expression vector.

Plasmid vectors for mutant CPYs—C193A, C207A, C262A, and C268A—were constructed using the QuickChange site-directed mutagenesis method (Agilent Technologies, Santa Clara, CA, USA). This method utilized the constructed recombinant wild-type CPY expression vector as a template with the following oligonucleotide primer pairs:

- **C193A-F:** 5'-GAACCAATGGCCGCTGGTGAAG GTG-3'
- **C193A-R:** 5'-CACCTTCACCAGCGCCATTGG TTC-3'
- **C207A-F:** 5'-CCCTCGGAGGAAGCTCTCTGCTAT GGAA-3'
- **C207A-R:** 5'-TTCCATAGCAGGCTCTCCGAGGG-3'
- **C262A-F:** 5'-CAGGAAGGATGCTGAAGGTGGGCAA-3'
- **C262A-R:** 5'-TTGCGACCTCCACGATCCTTCTCTT G-3'
- **C268A-F:** 5'-GTTGGCAAATTGGGCCTACCGA CGT-3'
- **C268A-R:** 5'-ACGTTGGGTAGGCAAATTGC- CACC-3'

The double-underscores denote the mutation sites. All the primers were synthesized by Eurofins Genomics (Tokyo, Japan). Each mutation introduced into the target site was confirmed by DNA sequencing that was performed at Fas-mac (Atsugi, Japan).

**Expression and purification of recombinant wild-type and mutant CPYs**

All mutants were expressed and purified by the same method for the recombinant wild-type CPY preparation. Saccharomyces cerevisiae BY4741Δprc1A (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, and prc1Δ::kanMX4) purchased from Thermo Fisher Scientific (Waltham, MA, USA) was transformed with the constructed plasmid vector using the lithium acetate method [16]. Transformants were selected on SD-Ura agar plate containing 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, 2% (w/v) agar, 30 µg·mL⁻¹ L-leucine, 20 µg·mL⁻¹ L-histidine monohydrochloride monohydrate, 30 µg·mL⁻¹ adenine hemisulfate, and 20 µg·mL⁻¹ L-methionine. The selected yeast cells were grown by shaking in YPD liquid medium containing 2% (w/v) polypeptide, 1% (w/v) yeast extract, and 2% (w/v) glucose at 28°C until the optical density at 600 nm exceeded 1.0. The culture was incubated for an additional 72 h at 20°C to induce the expression of recombinant CPY. Cells were harvested by centrifugation at 2000 g, and the pellet was stored at −30°C until used.

Purification of each CPY was performed as described previously [17]. The harvested cells were autolyzed with chloroform and water and centrifuged. The supernatant was collected, ammonium sulfate fractionation was carried out at 20%, and precipitates were removed. A second ammonium sulfate fractionation was carried out to 90%, and the precipitated proteins were collected. The precipitate was dissolved in 50 mM sodium acetate buffer, pH 5.0, and incubated at room temperature for 18 h so that overexpressed CPY protein was completely matured. After dialysis against 10 mM sodium phosphate buffer at pH 7.0, mCPY was purified by TOYOPEARL® Butyl-650M column chromatography (1.5 cm × 5 cm; Tosoh, Tokyo, Japan).

Each cell that expressed C193A or C207A mutant protein was also homogenized using Mini-Beadbeater (BioSpec Products, Bartlesville, OK, USA) to confirm the influence of the method of cell lysis for aggregation.

**SDS/PAGE and western blotting**

Expression and purification of the recombinant CPYs were confirmed by SDS/PAGE and western blotting. The rabbit serum containing polyclonal anti-CPY antibody (1 : 7500)
was prepared in our laboratory. Horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:100 000) was purchased from GE Healthcare (Buckinghamshire, UK).

**Protein assay**

The protein concentration of each solution in the extraction and purification steps was estimated using the Quick Start Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the standard protein. After the color development, protein contents were determined photometrically.

**Activity assays**

Peptidase activity of each purified recombinant CPY was assayed by incubating with N-benzyloxy carbonyl-L-phenylalanyl-L-leucine (Z-Phe-Leu-OH; Bachem, Bubendorf, Switzerland) at pH 7.0 and room temperature. L-Leucine, a hydrolysis product, was measured by amino acid analysis using the LaChromUltra U-HPLC system (Hitachi High-Technologies, Tokyo, Japan). Anilidase activity of each CPY was determined spectrophotometrically [17]. The release of p-nitroaniline from N-benzoyl-L-tyrosine p-nitroanilide (Bz-Tyr-pNA; Sigma-Aldrich, St. Louis, MO, USA) at pH 7.0 and room temperature was monitored at 410 nm using a model U-2000A spectrophotometer (Hitachi High-Technologies).

**Results**

**Expression patterns of recombinant mutant CPYs**

Expression of each recombinant mutant CPY was confirmed. After the inducible cultivation, yeast cells were directly lysed in the SDS/PAGE sample buffer and analyzed by western blotting with anti-CPY antiserum. Western blotting revealed that each mutant CPY was expressed in the yeast cells as a 69-kDa precursor form similar to the wild-type CPY (data not shown). This analysis did not provide information about protein folding, solubility was examined after autolysis by chloroform and water. C193A and C207A were recovered in the insoluble fraction, while C262A and C268A were in the soluble fraction (Fig. 2A,C). We also employed the bead beater method of homogenization for C193A and C207A and detected both mutant proteins in the precipitates using western blotting (Fig. 2B). As SDS/PAGE and western blot analyses of the solubilized whole-cell lysates detected the main band at ~69 kDa, it is likely that the recombinant protein was expressed. Both C193A and C207A mutants were found in the insoluble fraction after autolysis or bead beater treatment. Thus, these mutant proteins were likely aggregated during the protein extraction process. As the C193A and C207A mutants were unable to form disulfide bonds, some fluctuation would be introduced at the V-shape helix. This conformational instability might be the reason for the insoluble behavior during cell disruption and/or protein folding. C262A and C268A were purified as soluble proteins (Fig. 2C). The collective findings suggest that the disulfide bond at C193–C207 could be related to maintenance of the structure of the V-shape helix.
Hydrolase activity of mutant CPYs

Catalytic activity is an excellent index for the structural change occurring in CPYs [18]. The importance of disulfide bonds to the V-shape helix was evaluated by measuring enzyme activity. As C193A and C207A proteins failed to yield a soluble enzyme, we only examined C262A and C268A. Hydrolase activity was measured using synthetic substrates, where peptidase activity was determined by measuring the release rate of L-leucine from Z-Phe-Leu-OH, and anilidase activity was determined by measuring the release rate of p-nitroaniline from Bz-Tyr-pNA. Table 1 summarizes the hydrolase activities for wild-type CPY, C262A, and C268A. When peptidase activities are compared between mutant and wild-type, C262A and C268A reduced activities to 8.5% and 16% that of the wild-type, respectively. Similarly, anilidase activities were reduced to 1.8% and 1.9%, respectively. The results indicate that the disruption of C262–C268 affects the catalytic activity of CPY, where anilidase activity is more susceptible than peptidase activity.

Table 1. Relative specific activity of wild-type and soluble mutant CPYs toward Z-Phe-Leu-OH and Bz-Tyr-pNA

| CPY       | Z-Phe-Leu-OH | Bz-Tyr-pNA |
|-----------|--------------|------------|
| WT (100)  | (100)        |            |
| C262A     | 8.5          | 1.8        |
| C268A     | 16           | 1.9        |

* Enzyme reaction was performed with 1 mM Z-Phe-Leu-OH at pH 7.0 and room temperature. The amount of released L-leucine was determined by U-HPLC.; b Enzyme reaction was performed with 0.3 mM Bz-Tyr-pNA at pH 7.0 and room temperature, and an absorbance at 410 nm was monitored spectrophotometrically.

Fig. 3. Three-dimensional structural comparison of serine carboxypeptidases: CPY, CPWII (PDB ID: 3SC2[12]), and PPCA (PDB ID: 4MWS [24]). (A) Close-up view of the beginning and end of the V-shape helix or corresponding helical region of CPY, CPWII, and PPCA. The V-shape helix of CPY and corresponding helical regions of the other two enzymes are colored green. C193–C207 is a CPY-specific disulfide bond, and disulfide bonds corresponding to C262–C268 of CPY are identified in CPWII and PPCA. (B) Homodimer structure and the dimer interface of PPCA. Each core domain is colored light green and light blue, and the helical region is colored green and blue. In PPCA, the disulfide bond corresponding C193–C207 of CPY was not formed in the loop region at the beginning of the helical region. The residues of this loop region (196–200, colored pink) interact with the residues of paired subunit (colored light blue) by forming hydrogen bonds (red broken line) and hydrophobic interaction (blue broken line). Those intramolecular interactions were monitored using Accelrys Discovery Studio Visualizer 4.0.
Discussion

Contribution of the V-shape helix to CPY catalysis

The insertion domain in lipases and acetylcholinesterases, which is present in most members of the α/β hydrolase fold family, has a unique catalytic role [7–10]. CPY and related serine carboxypeptidases, which belong to this α/β hydrolase fold family, have a corresponding helical insertion domain, although the involvement of insertion domain in the catalytic function is not clearly defined. This study intended to reveal a possible role(s) of the insertion domain in CPY by disrupting two sets of disulfide bonds that support the helical structure (the V-shape helix) in the insertion domain. When the disulfide bond of C262–C268 was disrupted, the mutant enzyme lost both peptidase and anilidase activities (Table 1). A C262–C268 bridge located on the flexible loop at the exit side of the V-shape helix may contribute significantly to the catalytic process, probably by stabilizing the V-shape helix from unnecessary fluctuation during the catalysis.

The substrate-binding sites, S1 and S2–S5, and the hydrogen bond network located at the bottom of the S1′ site participate in substrate recognition [19–23]. Indeed, some amino acid residues at the V-shape helix comprise part of those substrate-binding sites. Our previous findings support the idea that the V-shape helix participates in the catalysis because the replacement of the residues at or near the V-shape helix influences the hydrolase activity of CPY [14]. To further elucidate this function of the V-shape helix, dynamic analyses, such as molecular dynamic simulations or kinetic analyses, would be required. It is important to note that a disulfide bridge equivalent to C262–C268 is present in both CPWII and PPCA. CPY and other serine carboxypeptidases differ slightly, in that the loop region in CPY bridged by the disulfide bond is short, whereas in CPWII and PPCA, the same region is long and a portion can be cleaved during maturation [12,13,24]. Therefore, it seems likely that the V-shape helix is necessary not only for catalysis, but also to maintain the active mature form, especially in the presence of C262–C268 or a corresponding bond.

Relationship between the V-shape helix and structural stability in CPY

In some cases, disulfide bond(s) help maintain protein conformation. Any disulfide bond disruption eventually leads to destabilization of protein structure. In yeast, such destabilized proteins are eliminated by the endoplasmic reticulum-associated degradation (ERAD) pathway, which efficiently recognizes and disposes of abnormal proteins [25]. One well-known example is a mutated CPY [26,27]. If recombinant CPYs are unfolded or misfolded in the ER, they become a target for ERAD. Similarly, mutant CPYs with disrupted disulfide zipper at C217–C240 and C224–C233 are susceptible for proteolysis [11]. As shown in Fig. 2A, recombinant C193A and C207A mutants were insoluble, but were not fragmented in yeast host cells. This indicates that the mutants were not a target of the ERAD pathway. The maintenance of the V-shape helix by the C193–C207 disulfide bridge may be important for the stable preservation of the folded CPY structure.

A comparison between the structures of CPY and other homologous serine carboxypeptidases CPWII and PPCA did not reveal a disulfide bond corresponding to C193–C207 in CPY (Fig. 3A). As C193–C207 contributes to the stability of CPY, there should be a different mechanism to replace the role of C193–C207 in both CPWII and PPCA. A hint to this mechanism is that both CPWII and PPCA are homodimers [12,13,24]. Two subunits are associated with each other around the insertion domain where the loop region is included at this interface (Fig. 3B). It seems probable that either the C193–C207 disulfide bridge or dimer formation contributes to a flexible loop structure at the proper place. However, the details remain to be determined.

The present results demonstrate that the V-shape helix in CPY is involved in its catalysis and maintenance of conformation. These findings can be extended to other serine carboxypeptidases belonging to the α/β hydrolase fold family. The insertion domain of this family is quite diverse. This diversity is reflected in the wide range of catalytic properties that have been observed for serine proteases [6,28]. We expect that further studies on the insertion domains will unravel the mystery of evolution and differentiation of α/β hydrolase fold family enzymes.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

HU conceived and directed the study. MM designed and performed the experiments. TS and NM provided the yeast expression vectors. MM wrote the manuscript. TS, NM, and HU revised the manuscript.
References

1 Hayashi R, Moore S and Stein WH (1973) Carboxypeptidase from yeast. Large scale preparation and the application to COOH-terminal analysis of peptides and proteins. *J Biol Chem* 248, 2296–2302.

2 Hayashi R and Hata T (1972) Action of yeast proteinase C on synthetic peptides and poly-α, L-amino acids. *Biochim Biophys Acta* 263, 673–679.

3 Hayashi R, Bai Y and Hata T (1975) Kinetic studies of carboxypeptidase Y. I. Kinetic parameters for the hydrolysis of synthetic substrates. *J Biochem* 77, 69–79.

4 Bai Y, Hayashi R and Hata T (1972) Action of yeast carboxypeptidase Y. III. Action on ester, amide, and amilide substrates and the effects of some environmental factors. *J Biochem* 78, 617–626.

5 Endrizzi JA, Breddam K and Remington SJ (1994) 2.8Å structure of yeast serine carboxypeptidase. *Biochemistry* 33, 11106–11120.

6 Ollis DL, Cheah E, Cygler M, Dijkstra B, Frolow F, Franken SM, Harel M, Remington SJ, Silman I, Schrag J, et al. (1992) The α/β hydrolase fold. *Protein Eng* 5, 197–211.

7 Brzozowski AM, Derewenda U, Derewenda ZS, Dodson GG, Lawson DM, Turkenburg JP, Bjorkling F, Huge-Jensen B, Patkar SA and Thim L (1991) A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. *Nature* 351, 491–494.

8 Derewenda U, Brzozowski AM, Lawson DM and Derewenda ZS (1992) Catalysis at the interface: the anatomy of a conformational change in a triglyceride lipase. *Biochemistry* 31, 1522–1541.

9 Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Toker L and Silman I (1991) Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototyptic acetylcholine-binding protein. *Science* 253, 872–879.

10 Tan RC, Truong TN, McCammon JA and Sussman JL (1993) Acetylcholinesterase: electrostatic steering increases the rate of ligand binding. *Biochemistry* 32, 401–403.

11 Maki T, Kozawa H, Mima J, Ueno H and Hayashi R (2002) Increased proteolytic susceptibility of carboxypeptidase Y caused by modification of the disulfide zipper. *Biosci Biotechnol Biochem* 66, 1393–1395.

12 Liao DI, Breddam K, Sweet RM, Bullock T and Remington SJ (1992) Refined atomic model of wheat serine carboxypeptidase II at 2.2Å resolution. *Biochemistry* 31, 9796–9812.

13 Rudenko G, Bonten E, d’Azzo A and Hol WG (1995) Three-dimensional structure of the human ‘protective protein’: structure of the precursor form suggests a complex activation mechanism. *Structure* 3, 1249–1259.

14 Makino M, Sahara T, Morita N and Ueno H (2014) Amino acid substitution reveals the role of V-shape helix on construction of yeast carboxypeptidase Y. *J Biol Macromol* 14, 27–41.

15 Sahara T, Goda T and Ohgiya S (2002) Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. *J Biol Chem* 277, 50015–50021.

16 Ito H, Fukuda Y, Murata K and Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* 153, 163–168.

17 Hayashi R (1976) Carboxypeptidase Y. *Methods Enzymol* 45, 568–587.

18 Kinsho T, Ueno H, Hayashi R, Hashizume C and Kimura K (2002) Sub-zero temperature inactivation of carboxypeptidase Y under high hydrostatic pressure. *Eur J Biochem* 269, 4666–4674.

19 Sorensen SB, Raaschou-Nielsen M, Mortensen UH, Remington SJ and Breddam K (1995) Site-directed mutagenesis on (serine) carboxypeptidase Y from yeast. The significance of Thr60 and Met398 in hydrolysis and aminolysis reactions. *J Am Chem Soc* 117, 5944–5950.

20 Olesen K, Mortensen UH, Aasmul-Olsen S, Kielland-Brandt MC, Remington SJ and Breddam K (1994) The activity of carboxypeptidase Y toward substrates with basic Pα amino acid residues is drastically increased by mutational replacement of leucine 178. *Biochemistry* 33, 11121–11126.

21 Mortensen UH, Remington SJ and Breddam K (1994) Site-directed mutagenesis on (serine) carboxypeptidase Y. A hydrogen bond network stabilizes the transition state by interaction with the C-terminal carboxylate group of the substrate. *Biochemistry* 33, 508–517.

22 Olesen K, Meldal M and Breddam K (1996) Extended substrate characterization of carboxypeptidase Y using substrates based on intramolecularly quenched fluorescence. *Protein Pept Lett* 3, 67–74.

23 Nakase H, Murata S, Ueno H and Hayashi R (2001) Substrate recognition mechanism of carboxypeptidase Y. *Biosci Biotechnol Biochem* 65, 2465–2471.

24 Kolli N and Garman SC (2014) Proteolytic activation of human cathepsin A. *J Biol Chem* 289, 11592–11600.

25 Meusser B, Hirsch C, Jarosch E and Sommer T (2005) ERAD: the long road to destruction. *Nat Cell Biol* 7, 766–772.

26 Wolf DH and Fink GR (1975) Proteinase C (carboxypeptidase Y) mutant of yeast. *J Bacteriol* 123, 1150–1156.

27 Finger A, Knop M and Wolf DH (1993) Analysis of two mutated vacuolar proteins reveals a degradation pathway in the endoplasmic reticulum or a related compartment of yeast. *Eur J Biochem* 218, 565–574.

28 Rauwerdink A and Kazlauskas RJ (2015) How the same core catalytic machinery catalyzes 17 different reactions: the serine-histidine-aspartate catalytic triad of α/β-hydrolase fold enzymes. *ACS Catal* 5, 6153–6176.