J. Biol. Macromol., **14**(1), 27-41 (2014)

Regular article

Amino acid substitution reveals the role of V-shape helix on construction of yeast carboxypeptidase Y

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Received January 28, 2014; Accepted February 4, 2014

Carboxypeptidase Y (CPY) is a vacuolar serine-type protease of yeast *Saccharomyces cerevisiae* to release wide variety of L-amino acid including proline from C termini of peptides and proteins. According to three-dimensional structure of mature CPY determined crystallographically, there is a characteristic region constructed with two adjacent α-helices, called 'V-shape helix', which lies over the active site cavity just like a door. In order to clarify the role of V-shape helix, we attempted to immobilize the V-shape helix by introducing amino acid substitution to create additional disulfide bridge between the V-shape helix and the main body of CPY. Resulting mutant enzymes presented with misfolding or hydrolase activity loss, suggesting that replacement of the residues at or near V-shape helix would influence on the protein folding and enzyme activity. The results of this report demonstrated that the insertion domain including V-shape helix in α/β hydrolase-fold serine carboxypeptidases plays a role in the construction of functional enzyme.

Key words: carboxypeptidase, serine protease, α/β hydrolase fold, protein folding, substrate recognition

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Introduction

Carboxypeptidase Y (CPY) is a vacuolar serine-type protease of yeast *Saccharomyces*

cerevisiae to hydrolyze esters, amides, and anilides, not only to release wide variety of L-amino acid including proline from C termini of peptides and proteins (1-4). The mature form of CPY exists as a 61-kDa monomer including 421 amino acid residues. Three-dimensional structure of mature CPY was determined by X-ray crystallography (5). It is suggested that the structure belongs to a group of α/β hydrolase-fold family which includes serine carboxypeptidase II from wheat (CPWII) and lipase from *Geotrichum candidum* (6-8). The conformation of α/β hydrolase fold proteins can be divided into two domains: core domain that is generally similar among the fold family and insertion domain that is composed of diverse structure elements. The residues 180~317 of CPY correspond to the insertion domain.

CPY has a characteristic region (residues 204~251) constructed with two adjacent α-helices, called 'V-shape helix', that is a part of the insertion domain (Fig. 1) (5). V-shape helix is common among serine carboxypeptidases, those having α/β hydrolase fold to include CPY, CPWII, and human protective protein/ cathepsin A (PPCA), where CPY has the largest V-shape helix (6,9). According to the crystal structure of mature CPY, V-shape helix lies over the active site cavity in which the essential catalytic triad, Ser146, His397, and Asp338, are positioned (5,10-12). Two disulfide bonds, Cys193-Cys207 and Cys262-Cys268, are located at the beginning and ending of the V-shape helix, just like hinges. Thus, it is projected that V-shape helix may move like a door during the catalysis. Most lipases exhibit an α/β hydrolase fold and also have characteristic structural feature, which is constructed with a mobile helical fragment, named lid or flap, and this part participates in controlling the access of substrate to the active site (13-17). However, in the carboxypeptidases, the motion of V-shape helix has not been discussed so far.

CPY utilizes C-terminal carboxylate and side chains of the substrates for the substrate recognition where a hydrogen bond network and substrate binging sites, S_1' and S_1~S_5 subsites, respectively, play critical roles (5,18-24). Interestingly, a part of V-shape helix constru cts the S_3 and S_5 subsites. Hence, it is reasonable to assume that V-shape is involved in the substrate recognition mechanism by approaching to the active site cavity.

In this study, it is of our aim to clarify the role of V-shape helix in the construction of CPY. In order to verify the involvement of V-shape helix in the substrate accessing, we attempted to immobilize the V-shape helix by introducing amino acid substitutions, so that additional disulfide bridge between the V-shape helix and the core domain of CPY would be created. Two pairs of nearest

residues, Tyr225 and Pro315, and Gln228 and Lys314, were selected for the mutation sites based upon the distance of Cβ atoms between the residues on V-shape helix and that on core domain, where distances for Tyr225-Pro315 and Gln228-Lys314 are 4.5 Å and 5.8 Å, respectively (Fig. 2). All these residues seem to be unrelated to the substrate accessing and recognition. Double mutants, Y225C/P315C and Q228C/K314C, and single mutants, Y225C, P315C, Q228C, and K314C were produced by using a novel yeast expression system. Also constructed were the mutants, Y225A, Y225F, P315S, and P315G, in order to verify the effects of the mutation at or near V-shape helix on protein expression and enzyme activity of CPY.

Materials and methods

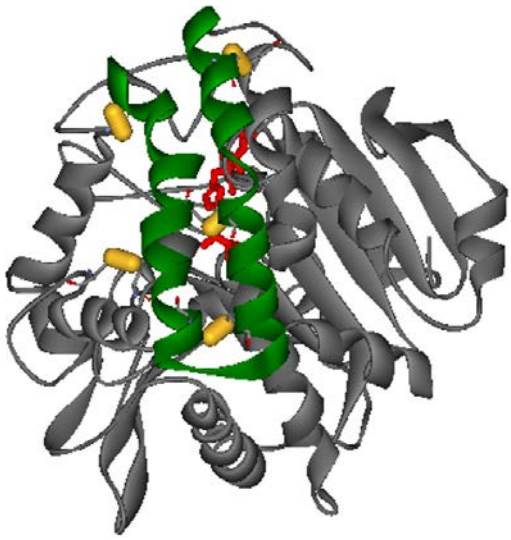

Fig. 1 Three dimensional structure of mature CPY determined by X-ray crystallography (PDB ID: 1YSC)
V-shape helix (Ser204~Thr251) is colored in green. The catalytic triad (Ser146, His397, and Asp338) are shown in red stick. Disulfide bonds are shown in orange thick stick.

Materials

S. cerevisiae BY4741*prc1Δ* (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, prc1Δ::kanMX4*) was obtained from Thermo

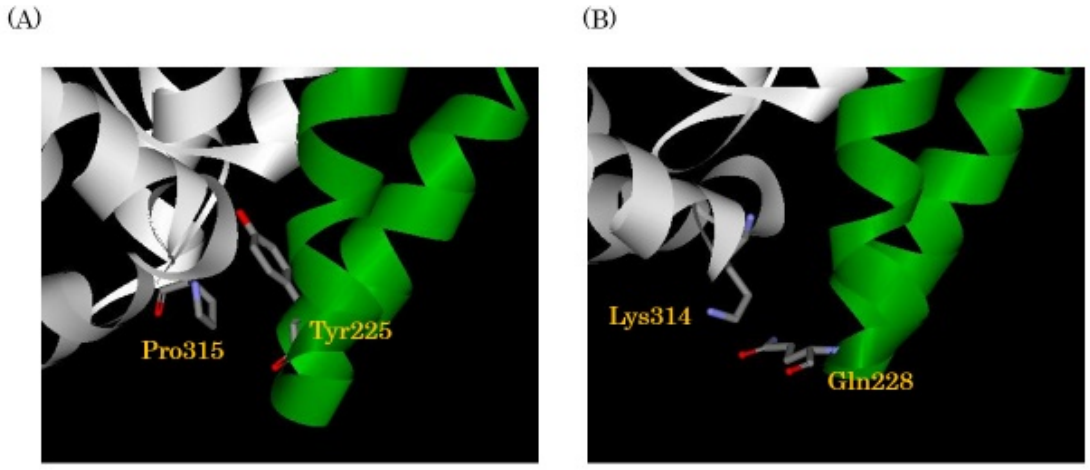

Fig. 2 Position of residues replaced by Cys
Two pairs of residues (Tyr225 and Pro315 (A), and Gln228 and Lys314 (B)) were selected for replacement by Cys. The distance of Cβ atoms of Tyr225 and Pro315 was 4.5 Å and that of Gln228 and Lys314 was 5.8 Å. V-shape helix was colored in green.

Fisher Scientific (Waltham, MA, USA). Cloning vector pGEM-T Easy Vector was purchased from Promega (Madison, WI, USA). QuikChange Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (Santa Clara, CA, USA). Restriction enzymes used in this study were purchased from Takara Bio (Shiga, Japan), Toyobo (Osaka, Japan), and Roche (Basel, Switzerland). T4 DNA ligase was obtained from Takara Bio. Blend Taq and *Taq* DNA Polymerase were purchased from Toyobo and New England Biolabs (Ipswich, MA, USA), respectively. TOYOPEARL Butyl-650M resin for hydrophobic interaction chromatography was obtained from Tosoh (Tokyo, Japan). Quick Start Bradford Protein Assay Kit was purchased from Bio-Rad (Hercules, CA, USA). N-benzoyl-L-tyrosine *p*-nitroanilide (BT*p*NA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Benzyloxycarbonyl-L-phenilalanyl-L-leucine (Z-Phe-Leu-OH) and benzyloxycarbonyl-L-phenilalanyl-L-proline (Z-Phe-Pro-OH) were obtained from Bachem (Bubendorf, Switzerland), and N-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) was obtained from Peptide Institute, Inc. (Osaka, Japan). Synthetic oligonucleotide primers were purchased from Japan Bio Services (Saitama, Japan) and Operon Biotechnologies (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Nakarai Tesque (Kyoto, Japan).

Plasmid constructions and site-directed mutagenesis

The sequence of CPY was obtained from Saccharomyces genome database (http://www.yeastgenome.org/). Molecular modeling of mature CPY was carried out with Discovery Studio 2.5.5 (Accelrys, San Diego, CA, USA) using crystal structure of mature CPY (PDB ID: 1YSC). Genomic DNA was extracted from *Saccharomyces cerevisiae* wild-type strain using potassium acetate method (25). *Prc1* fragment encoding full-length wild-type CPY was amplified from genomic DNA by PCR with Blend Taq DNA polymerase and oligonucleotide primer pair, SmaI-prc1-F and prc1-XhoI-R (Table 1). In this PCR, *Sma*I restriction site and *Xho*I restriction site were introduced to the 5'- and 3'- termini of *prc1*, respectively. The PCR

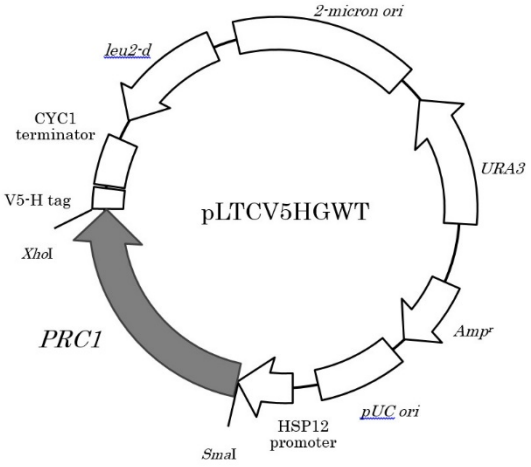

Fig. 3 Schematic diagram of the constructed plasmid vector pLTCV5HGWT

product was inserted into pGEM-T easy vector and constructed pGCWT. Then pGCWT was digested with *Sma*I and *Xho*I and the fragment was ligated into pLTex321sV5H that was digested with *Sma*I and *Xho*I. Resulting recombinant CPY expression plasmid vector pLTCV5HGWT, a novel yeast low-temperature inducible expression vector, was thus constructed (Fig. 3).

Plasmid vectors for CPY variants were constructed by using a QuikChange Site-Directed Mutagenesis method employing pLTCV5HGWT as the template and each oligonucleotide primer pair as shown in Table 1. Insertion of *prc1* fragment to the plasmid vector and each mutation introduced into the target sites were confirmed by DNA sequencing entrusted to Fasmac (Kanagawa, Japan).

Expression and purification of recombinant wild-type and mutant CPYs

Tyr225 and Pro315 substituted mutant proteins were expressed and purified by the same method for the recombinant wild-type CPY preparation as follows: *Saccharomyces*

Table 1 Oligonucleotide primers using for construction of plasmid vectors of wild-type CPY, Cys-introduced mutants (Y225C, P315C, Y225C/P315C, Q228C, K314C, and Q228C/K314C) and Tyr225- and Pro315-substituted mutants (Y225A, Y225F, P315S, and P315G) by site-directed mutagenesis method. SmaI-prc1-F and prc1-XhoI-R were used for amplifying *prc1* fragment. *Sma*I restriction site and *Xho*I restriction site are underlined, respectively. The other primers were used as forward primers for DNA mutagenesis. The sequence of each forward primer is only shown. Mutation sites are indicated by double-underlines.

Primers	Sequences
SmaI-prc1-F	5'-ACACCCGGGATGAAAGCATTC-3'
prc1-XhoI-R	5'-ACGCTCGAGTAAGGAGAAACC-3'
Y225C-F	5'-TCGAGTCGTGCTGTGACTCGCAATC-3'
P315C-F	5'-GATTGGATGAAGTGTTACCACACCGC-3'
Q228C-F	5'-GTGCTATGACTCGTGCTCCGTCTGGTCC-3'
K314C-F	5'-GGGTGATTGGATGTGTCCTTACCACACCG-3'
Y225A-F	5'-TCGAGTCGTGCGCTGACTCGCAATC-3'
Y225F-F	5'-TCGAGTCGTGCTTTGACTCGCAATC-3'
P315S-F	5'-GATTGGATGAAGTCTTACCACACCGC-3'
P315G-F	5'-GATTGGATGAAGGGTTACCACACCGC-3'

cerevisiae BY4741*prc1Δ* (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, prc1Δ::kanMX4*) was transformed with expression vector using lithium acetate method (26). The transformant was selected on SD-ura plate medium, contained 0.67% Yeast Nitrogen Base without Amino Acids, 2% glucose, 2% agar, 30 μg/ml L-leucine, 20 μg/ml histidine monohydrochloride monohydrate, 30 μg/ml adenine hemisulfate dehydrate, and 20 μg/ml L-methionine. The selected yeast cell was grown in YPD medium, contained 2% polypeptone, 1% yeast extract, and 2% glucose at 28 °C until OD_{600} reached over 1.0. The temperature was lowered at 20 °C and the culture was shaken for 3 days in order to induce recombinant wild-type CPY or mutant protein expression. The cells were harvested by centrifugation at 2,000 ×g.

Purification of each recombinant CPY was performed according to the method described previously (27). The cell pellet was mixed with 0.2 ml/g wet cell of chloroform and stirred at room temperature for 30 min. Then 0.4 ml/g wet cell of H_2O was added and the pH of the mixture was adjusted to 7.0 and was kept stirred at room temperature for 2 h. The pH was readjusted to 7.0 and the mixture was left at room temperature for 16 h under stirring. The lysate was centrifuged at 5,000 ×g and ammonium sulfate fractionation was carried out for the supernatant first at 20% then at 90%. The precipitates were dissolved in 3 ml/g wet weight of 50 mM sodium acetate buffer, pH 5.0, and adjusted to pH 5.0 and incubated at room temperature for 18 h to activate precursor CPY. The activated solution was dialyzed against 10 mM sodium phosphate buffer, pH 7.0.

Activated CPY was purified from the dialyzed solution by hydrophobic interaction chromatography (HIC) on TOYOPEARL Butyl-650M. The solution was diluted 1:5 with 10 mM sodium phosphate buffer, pH 7.0, containing 2 M ammonium sulfate and applied to a column of TOYOPEARL Butyl-650M (1.5 × 5 cm) equilibrated with the same buffer. The column was washed with 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M ammonium sulfate and then the eluate with 10 mM sodium phosphate buffer (pH 7.0) was collected.

The eluate was concentrated by ultrafiltration and treated at 50 °C for 10 min. After centrifugation at 10,000 ×g, the supernatant was pooled.

Protein concentration

Protein concentration in each enzyme solution was determined by Quick Start Bradford Protein Assay Kit (Bio-Rad) using bovine serum albumin as a standard protein.

SDS-PAGE and Western blotting

The expression of recombinant CPYs and protein purity of each purification step were

evaluated by SDS-PAGE and Western blotting. Polyacrylamide gel at 10% containing 0.1% SDS was used for SDS-PAGE and the gel was stained with 0.1% (w/v) CBB R-250 contained dye after electrophoresis. In Western blotting, the proteins within the SDS-PAGE gel were transferred to PVDF membrane electrically with Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) with the use of CAPS buffer, pH 11. After blocking with skim milk at room temperature for 1 h or at 4 ℃ overnight, the membrane was incubated with rabbit polyclonal anti-CPY antiserum (1:10,000) prepared in our laboratory and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (1:100,000) (GE Healthcare, Buckinghamshire, UK) in turn to detected mature wild-type CPY and mutant proteins. The immuno-positive bands were visualized with ImmunoStar LD (Wako) and were exposed on an instant film using ECL Mini-camera (GE Healthcare).

Kinetic analyses of hydrolysis activities of WT and mutant enzymes

Anilidase activities of wild-type CPY and soluble mutant enzymes toward BT*p*NA were determined spectrophotometrically (27,28). The release of *p*-nitroaniline from BT*p*NA at pH 7.0 was monitored at 410 nm at room temperature using U-2000A Spectrophotometer (Hitachi High-Technologies, Tokyo, Japan). Peptidase activities of those enzymes were measured by amino acid analysis using LaChromUltra U-HPLC system (Hitachi High-Technologies) for the increase of L-leucine or L-proline at pH7.0, products of the hydrolyses of Z-Phe-Leu-OH or Z-Phe-Pro-OH, respectively, at room temperature. Esterase activities of WT and P315G were determined by the spectrophotometric method (27,29): Hydrolysis of Ac-Tyr-OEt at pH 8.0 was measured at 237 nm at room temperature. Kinetic parameters, k_{cat} and K_m, were calculated from Michaelis-Menten equation and Hanes-Woolf plots.

Results

Expression and purification of recombinant wild-type and mutant CPYs

The constructed plasmid, pLTCV5HGWT, was introduced into a *S. cerevisiae* strain BY4741*prc1Δ* and the transformant was cultured in YPD medium. Wild-type CPY was expressed inducibly by lowering the culture temperature from 28 to 20 ℃. Wet cell paste of 11.2 g was obtained from 1 l culture. Since the recombinant CPY was produced as an inactive precursor in the cells, they were extracted from the cells and were activated by the conventional method (27). After the activation, mature wild-type CPY

was detected on SDS-PAGE at approximately 61 kDa. After the heat treatment, WT was yielded with approximately 100-fold purification (Fig. 4 and Table 2).

Expression vectors for CPY variants were constructed by site-directed mutagenesis using pLTCV5HGWT as a template. DNA sequence analysis confirmed the codon changes. Each mutant protein was expressed in the transformed yeast cell and purified by the same method as WT preparation. Western blot analysis of the cell suspension of the transformants after inductive cultivation showed that all of cysteine introduced mutants were expressed in the yeast cells, respectively. However, Y225C, P315C, and double mutants, Y225C/P315C and Q228C/K314C, precipitated during the purification steps, while the other mutants, Q228C, and K314C, were recovered in soluble fractions and were purified (Fig. 5(A)). This result suggests that the precipitated mutant proteins tended to misfold easily. Both double mutants aggregated, suggesting that the excessive presence of cysteine at and around V-shape helix altered the protein folding.

The result that Q228C and K314C were purified as soluble proteins implies that substitution of Gln228 and Lys314 may not affect the protein folding. On the other hand, all of Tyr225 substituted mutants aggregated (Fig. 5(B)). This result suggests that tyrosine at 225 may be important for folding; hence, and V-shape helix plays a role in correcting folding process because Tyr225 is one of the component residues of V-shape helix. Among the mutants on Pro315, only P315C aggregated, while the other mutants, P315S and P315G, were purified as soluble proteins. Thus, Pro315 may not be involved

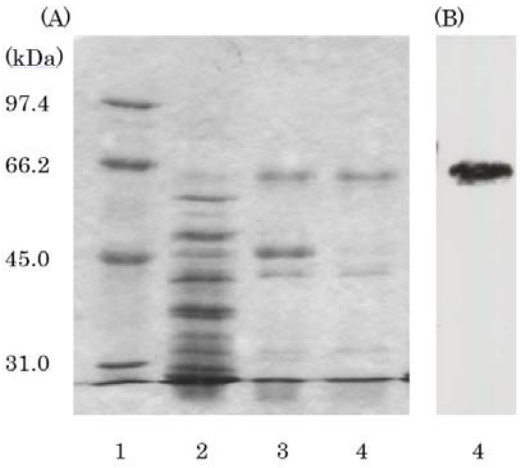

Fig. 4 SDS-PAGE (A) and Western blotting (B) of recombinant wild-type CPY at purification steps
Yeast cells in which recombinant WT is expressed were lysed by chloroform and the supernatant was treated with ammonium sulfate. Pro-CPY in the precipitates was activated at pH 5.0 and activated mature CPY was purified by hydrophobic interaction chromatography on TOYOPEARL Butyl-650M and heat treatment as described in the text. In Western blotting, rabbit polyclonal anti-CPY antiserum (1:10,000) and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (1:100,000) were used.
(A) (B) Lane 1, molecular marker (phosphorylase b, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa); lane 2, the solution after activation (31 μg); lane 3, the eluate from TOYOPEARL Butyl-650M column (3.2 μg); lane 4, the supernatant after heat treatment (2.6 μg).

Table 2 Summary of purification of recombinant wild-type CPY
Each parameter was calculated based on the anilidase activity toward BT*p*NA.

Steps	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Recovery (%)	Purification
Activation	70	360	5.1	100	1
Hydrophobic interaction chromatography	1.4	430	310	120	61
Heat treatment	0.64	350	550	97	110

in the folding; however, cysteine at this position could be an exception.

Hydrolase activity of soluble mutants

The peptidase and anilidase activities of the soluble proteins, Q228C and K314C, were measured. The activity toward Z-Phe-Leu-OH was determined by measuring the rate of release of L-leucine at room temperature. The anilidase activity was assayed at room temperature using BT*p*NA as a substrate by measuring the rate of release of *p*-nitroaniline. Compared with wild-type CPY, both mutants exhibited lower hydrolase activities toward Z-Phe-Leu-OH and BT*p*NA (Table 3(A)). This result suggests that the replacements of Gln228 and Lys314 would affect their hydrolase activities, while the protein folding was not influenced by the replacement.

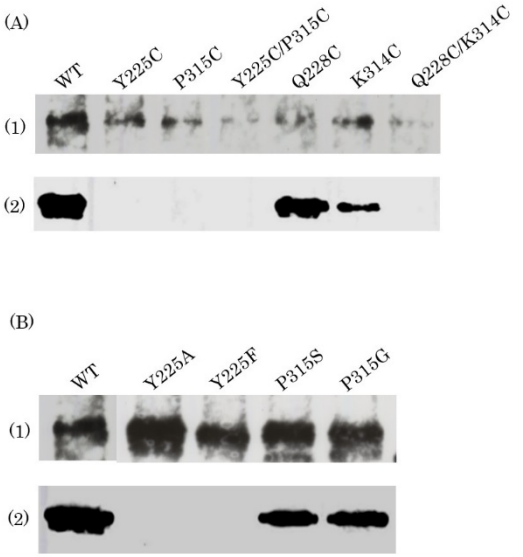

Fig. 5 Western blotting of mutant CPYs
(A) Cys-introduced mutants expressed in yeast cells (1) and activated and purified (2). (B) Tyr225- and Pro315-substituted mutants expressed in yeast cells (1) and activated and purified (2).

Table 3(B) shows the summary of hydrolase activities of wild-type CPY, P315S, and P315G. The relative ratios of the specific peptidase activities of P315S and P315G to that of WT were 51 and 55, respectively. This result indicates that P315S and P315G lose their peptidase activities toward Z-Phe-Leu-OH by half than that of WT. On the other hand, the relative ratio of specific anilidase activity of P315S and P315G to WT were 61 and 170, respectively. This result shows that the specific activity of P315G was slightly higher than that of WT, while that of P315S

decreased to approximately 60% of that of WT. Therefore, the replacement of Pro315 may influence catalysis and P315G may alter its substrate preference.

Kinetic analysis of P315G

In order to investigate catalytic activity of P315G in derails, kinetic analysis was performed. Table 4 shows the kinetic parameters for P315G-catalyzed hydrolyses of each substrate. Synthetic Z-dipeptides, Z-Phe-Leu-OH and Z-Phe-Pro-OH, were used for evaluating the hydrolysis activity of peptide substrates. The k_{cat}/K_m values of P315G for both substrates were significantly reduced as compared with wild-type CPY.

Table 3 Relative specific activity of WT and soluble mutant CPYs toward Z-Phe-Leu-OH and BT*p*NA
(A) Cys-introduced mutants (Q228C, K314C). (B) Pro315-subsituted mutants (P315S and P315G). The activities of recombinant WT for each substrate were set as 100% active. [a]Enzyme reaction was performed with 1 mM Z-Phe-Leu-OH at pH 7.0 at room temperature and the amount of released L-Leu was detected by U-HPLC. [b]Enzyme reaction was performed with 0.3 mM BT*p*NA at pH 7.0 at room temperature and A_{410} was monitored spectrophotometrically.

(A)

CPY	Z-Phe-Leu-OH[a]	BT*p*NA[b]
WT	(100)	(100)
Q228C	57	65
K314C	33	41

(B)

CPY	Z-Phe-Leu-OH[a]	BT*p*NA[b]
WT	(100)	(100)
P315S	51	61
P315G	55	170

The k_{cat} values of P315G for Z-Phe-Leu-OH and Z-Phe-Pro-OH were 3.2 and 0.23, respectively, while those of WT were 21 and 1.0, respectively. Thus, the decrease in k_{cat}/K_m of P315G for those substrates was ascribed to the decrease in k_{cat}.

In the hydrolysis of Ac-Tyr-OEt, the K_m value of P315G was slightly decreased relative to WT. Nevertheless, the catalytic efficiency of P315G was much lower than that of WT, due to its remarkable reduction of the k_{cat} value as compared with WT. On the other hand, the kinetic parameters of P315G for BT*p*NA were similar to those of wild-type CPY. These results show that the anilidase activity is maintained in P315G, although the catalysis of P315G in the hydrolysis of peptides and esters is significantly reduced in

Table 4 Kinetic parameters of recombinant wild-type CPY and P315G in the hydrolysis of the dipeptide substrates (A), anilide substrate (B), and ester substrate (C) at room temperature
Each parameter is expressed as mean ± SD calculated from the data of multiple experiments.

(A)

CPY	Z-Phe-Leu-OH		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
WT	15±1	0.067±0.011	230±21
P315G	2.0±0.5	0.019±0.005	100±2
CPY	Z-Phe-Pro-OH		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
WT	0.54±0.11	0.12±0.08	6.9±3.8
P315G	0.21±0.07	0.27±0.23	2.0±1.5

(B)

CPY	BT*p*NA		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
WT	0.57±0.04	0.038±0.002	15±0
P315G	0.40±0.05	0.031±0.004	13±2

(C)

CPY	Ac-Tyr-OEt		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}\cdot s^{-1}$)
WT	160±33	1.8±0.2	90±8
P315G	7.8±5.7	0.39±0.35	36±17

comparison with wild-type enzyme.

Discussion

According to the refolding experiments, the propeptide region of CPY acts as an intramolecular chaperon and denatured pro-CPY is refolded with correct disulfide bond formation (30,31). Hence, CPY has a high ability to renature spontaneously. However, in this study, it was found that the presence of additional only one cysteine at or near the tip of V-shape helix caused a severe misfolding (Fig. 5(A)). Furthermore, our data presented in Fig. 5(B) that replacement of Tyr225 positioned at the tip of V-shape helix by other amino acids led to aggregation of CPY molecule show that the residue at the tip of the V-shape helix contribute to the formation of proper conformation of CPY. Thus, our results demonstrate that V-shape helix would be involved in the folding of CPY molecule.

To date, there has been little recognition of the residues that are involved in catalysis besides these residues at the substrate binding sites in CPY. On the other hand, experimental results of this study showed that the residues, Gln228, Lys314, and Pro315, which are positioned at or near V-shape helix and are distant away from the active site, are involved in the hydrolysis activity of CPY (Table 3(A)(B)). In combination with the facts that V-shape helix lies over the active site cavity and that S_3 and S_5 subsites consist of part of the residues at V-shape helix, our data could demonstrate the involvement of V-shape helix in catalysis.

In generally, proline and glycine residues act as secondary structure breakers: the side chain of proline restrains its backbone and proline has the lowest conformational entropy, and glycine has a high flexibility because of its hydrogen substituent (32-35). In this study, the various phenotypes of each mutant which Pro315 was replaced by other amino acid reveal that the fluctuation of the backbone of residue 315 caused by the substitution of Pro315 have apparent effects on the protein folding (Fig. 5(B)), hydrolase activity (Table 3(B)), and substrate recognition (Table 4). This fluctuation would influence the construction of V-shape helix because the residue 315 is proximate to V-shape helix. Thus, it is necessary for functional CPY to maintain properly the configuration of the amino acid residues around V-shape helix.

In conclusion, we propose that V-shape helix plays a role in constructing a functional CPY. And it was demonstrated that the insertion domain, part of structure elements that deviate from the core domain, is a key region in α/β hydrolase fold serine carboxypeptidase as well as lipases. This study will provide a new insight into the

folding process and diversity of substrate recognition mechanism in α/β hydrolase fold proteins.

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Communicated by Takahashi Saori