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Substrate Recognition Mechanism of Carboxypeptidase Y

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To clarify the substrate-recognition mechanism of carboxypeptidase Y, Fmoc-$(Glu)_n$Ala-OH (n = 1 to 6), Fmoc-$(Glu)_n$Ala-NH_2 (1 to 5), and Fmoc-Lys$(Glu)_3$Ala-NH_2 were synthesized, and kinetic parameters for these substrates were measured. K_m for Fmoc-peptides significantly decreased as peptide length increased from n = 1 to n = 5 with only slight changes in k_{cat}. K_m for Fmoc-$(Glu)_{5,6}$Ala-OH were almost the same as one for protein substrates described previously (Nakase *et al.*, *Bull. Chem. Soc. Jpn.*, 73, 2587-2590). These results show that the enzyme has six subsites (S_1' and S_1-S_5). Each subsite affinity calculated from the K_m revealed subsite properties, and from the differences of subsite affinity between pH 6.5 and 5.0, the residues in each subsite were predicted. For Fmoc-peptide amide substrates, the priorities of amidase and carboxamide peptidase activities were dependent on the substrate. It is likely that the interactions between side chains of peptide and subsites compensate for the lack of P_1'-S_1' interaction, so the amidase activity prevailed for Fmoc-$(Glu)_{3,5}$Ala-NH_2. These results suggest that these subsites contribute extensively to substrate recognition rather than a hydrogen bond network.

Key words: carboxypeptidase Y; Fmoc-peptide; Fmoc-peptide amide; subsite affinity; substrate recognition mechanism

Carboxypeptidase Y (CPase Y; EC 3.4.12.1) is a vacuolar serine carboxypeptidase from bakers' yeast, *Saccharomyces cerevisiae*. This enzyme catalyzes a stepwise removal of C-terminal amino acids from peptides and proteins.

Kinetic studies using synthetic *N*-acylated dipeptide substrates and crystallographic study have shown that CPase Y recognizes P_1' and P_1 amino acids of substrates by S_1' and S_1 subsites, respectively, and C-terminal carboxylate by a hydrogen bond network.[1-4] In our previous study, it was found the affinity of CPase Y to protein substrates is 10^2 to 10^3 times higher than that to synthetic *N*-acylated dipeptide substrates due to several additional subsites besides S_1' and S_1 subsites.[5] These subsites may also contribute to the broad specificity of CPase Y, but their properties have been unclear. Our concern is to discover the number of subsites and the properties of each subsite.

Many peptide hormones, neurotransmitters, and growth factors are amidated at their C-terminus[6] and the amide structure contributes to their stability and biological activity. Serine carboxypeptidases have been implicated in the turnover of biologically active peptide amides such as endothelin I and tachykinins.[7,8] CPase Y also releases C-terminal amino acid amides (carboxamide peptidase activity) and/or ammonia (amidase activity) from these C-terminal amidated peptides.[9-11] Therefore, CPase Y is thought to be involved in the *in vivo* degradation of peptide hormones that carry an amide group at their C-terminus. However, the recognition mechanism of the C-terminal part of peptide-amide substrates is not well known for CPase Y. Elucidation of this mechanism may give a clue to the actual role of CPase Y *in vivo*.

In this study, we have tried to determine the subsite number of CPase Y and the contribution of these subsites to substrate recognition by measuring kinetic parameters for several peptides and peptide amides. For this purpose, we synthesized a series of Fmoc-$(Glu)_n$Ala-OH (n = 1-6) and Fmoc-$(Glu)_n$Ala-NH_2 (n = 1-5) as the substrate of CPase Y using a solid phase peptide synthesizer. These substrates are considered suitable for kinetic experiments because CPase Y hardly catalyzes the hydrolysis of the peptide bond between Glu-Glu residues, and they are soluble due to charged residues. We also analyzed the properties of each subsite of CPase Y by calculating each subsite affinity from kinetic parameters.

Materials and methods

Materials. PAC-PEG-PS (peptide acid linked polyethylene-graft polystyrene supports, 0.17 mmol

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Abbreviations: A_n, subsite affinity at S_n subsite; CPase, carboxypeptidase; ΔA_n, difference of subsite affinity between glutamic acid and glutamate; Fmoc, 9-fluorenylmethoxycarbonyl; RCM, reduced and carboxymethylated; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate

Table 1. Amino Acid Compositions and Yields of the Purified Fmoc-peptides and Fmoc-peptide Amides

Peptide	Amino acids[a]						Yield (%)
	Glu		Ala		Lys		
	Obs.	Theor.	Obs.	Theor.	Obs.	Theor.	
Fmoc-EA-OH	0.9	1	1.1	1	0	0	54
Fmoc-EEA-OH	2.0	2	1.0	1	0	0	70
Fmoc-EEEA-OH	2.8	3	0.9	1	0	0	58
Fmoc-EEEEA-OH	4.2	4	1.0	1	0	0	60
Fmoc-EEEEEA-OH	5.1	5	1.0	1	0	0	56
Fmoc-EEEEEEA-OH	5.9	6	0.9	1	0	0	62
Fmoc-EA-NH_2	1.0	1	0.9	1	0	0	48
Fmoc-EEA-NH_2	1.9	2	1.1	1	0	0	58
Fmoc-EEEA-NH_2	2.9	3	1.0	1	0	0	56
Fmoc-EEEEA-NH_2	4.1	4	0.9	1	0	0	64
Fmoc-KEEEA-NH_2	2.9	3	1.0	1	1.1	1	62
Fmoc-EEEEEA-NH_2	5.2	5	0.9	1	0	0	70

[a] These values were measured by amino acid analysis after hydrolysis in 6 M HCl at 110°C for 20 h *in vacuo*.

equivalent/g) and Fmoc-PAL-PEG-PS (Fmoc-peptide amide linked polyethylene-graft polystyrene supports, 0.17 mmol equivalent/g) were purchased from PerSeptive Biosystems (Massachusetts, U.S.A.); 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and Fmoc-L-alanine from Watanabe Chemical Industry (Hiroshima, Japan); Fmoc-L-glutamic acid γ-*tert*-butyloxy ester (Peptide Institute, Osaka, Japan) and Fmoc-N$^{\varepsilon}$-*tert*-butyloxycarbonyl-L-lysine from Peptide Institute (Osaka, Japan). CPase Y and H397A mutant in which His397 of CPase Y was replaced with alanine by site-directed mutagenesis were prepared as described previously.[10,12]

Synthesis of Fmoc-peptides and Fmoc-peptide amides. The resins, 290 mg PAC-PEG-PS (0.05 mmol) and 290 mg Fmoc-PAL-PEG-PS (0.05 mmol), were used for synthesis of peptides and peptide amides, respectively. Attachment of Fmoc-Ala to PAC-PEG-PS was done by the methods described by Stewart and Young.[13] Fmoc-$(Glu)_n$Ala-OH (n = 1–6), and Fmoc-$(Glu)_n$Ala-NH_2 (n = 1–5) and Fmoc-$Lys(Glu)_3$Ala-NH_2, were synthesized by using TBTU as an activator on a Pioneer Peptide Synthesize System (PerSeptive Biosystems) with a standard peptide synthesis program. All synthetic peptides and peptide amides were cleaved from the solid supports by stirring with a cleavage mixture containing 8.6 ml of trifluoroacetic acid, 1.2 ml of thioanisol, and 0.2 ml of *m*-cresol at room temperature for 2 h. These were purified by a Shimadzu HPLC system using a Shimadzu C18 column 5C18-AR (4.6 × 150 mm) with a linear gradient (0–40% acetonitrile concentration) using a water-acetonitrile system. Amino acid compositions and yields of purified synthetic peptides and peptide amides were confirmed using an amino acid analyzer (JEOL JLC-500/V) after hydrolysis in 6 M HCl at 110°C for 20 h *in vacuo*. Yields of the purified peptides and peptide amides were 50–70% and their amino acid compositions were well consistent with their theoretical values (Table 1).

Kinetics for Fmoc-$(Glu)_n$Ala-OH. One milliliter mixture of Fmoc-$(Glu)_n$Ala-OH (0.1 to 5.5 μM) and CPase Y (1.6 nM) in 50 mM MES buffer (pH 5.0 or 6.5) was incubated at 25°C for 2 min. The reaction was stopped by an addition of 33 μl of 10% sulfosulicylic acid for a final concentration of 2.5%. After a brief centrifugation, the supernatant was obtained and adjusted to pH 2.2 by an addition of 5 M NaOH. The detection of the amino acid product was done with an amino acid analyzer (JEOL JLC-500/V). Kinetic parameters were calculated from Lineweaver-Burk plots.

Assuming that acylation is the rate-limiting step,[5] subsite affinities (A_n) from S_2 to S_6 of glutamic acid residues were calculated using the following equation based on the standard free energy change:

$$A_n = RT\ln\frac{K_m \text{ for Fmoc-}(Glu)_{n-1}\text{Ala-OH}}{K_m \text{ for Fmoc-}(Glu)_n\text{Ala-OH}} \quad (1)$$

$$\Delta A_n = A_n \text{ at pH } 6.5 - A_n \text{ at pH } 5.0 \quad (2)$$

where R is the gas constant, and T is the absolute temperature.

Hydrolysis of Fmoc-$(Glu)_n$Ala-NH_2 and Fmoc-$Lys(Glu)_3$Ala-NH_2. One milliliter mixture of Fmoc-$(Glu)_n$Ala-NH_2 (10 μM), and CPase Y (0.8 nM) in 50 mM MES buffer (pH 6.5) was incubated at 25°C for 5 min. The reaction mixture was treated as described above and released Ala-NH_2, ammonia, and Ala were detected by an amino acid analyzer for the kinetic characterization. Alanine amide was eluted between histidine and lysine on the amino acid analyzer.

Fluorescence of CPase Y in the presence and absence of Fmoc-$(Glu)_{4,5}$Ala-OH. A stable complex

Table 2. Kinetic Parameters of the CPase Y-Catalyzed Hydrolysis of Synthetic Fmoc-peptides at pH 6.5[a]

Substrate (Fmoc-(Glu)$_n$Ala-OH)	k_{cat} (sec-1)	K_m ($\times 10^{-7}$ M)	k_{cat}/K_m ($\times 10^4$ sec^{-1} M^{-1})
Fmoc-EA-OH	5.5±0.4	2100±300	2.6±0.3
Fmoc-EEA-OH	2.7±0.2	350±20	7.7±0.5
Fmoc-EEEA-OH	2.2±0.2	90±8	24±3
Fmoc-EEEEA-OH	2.5±0.4	25±3	100±20
Fmoc-EEEEEA-OH	2.3±0.3	1.3±0.2	1800±200
Fmoc-EEEEEEA-OH	2.3±0.2	1.2±0.3	1900±300

[a] The reaction was done in 0.05 M MES, pH 6.5, at 25°C for 2 min. k_{cat} and K_m values were measured at substrate concentrations ranging from 0.1 to 5.5 μM using 1.6 nM CPase Y. Only alanine was released under these conditions.

Table 3. Kinetic Parameters of the CPase Y-Catalyzed Hydrolysis of Synthetic Fmoc-peptide at pH 5.0[a]

Substrate (Fmoc-(Glu)$_n$Ala-OH)	k_{cat} (sec^{-1})	K_m ($\times 10^{-7}$ M)	k_{cat}/K_m ($\times 10^4$ sec^{-1} M^{-1})
Fmoc-EA-OH	6.7±0.6	240±20	28±2
Fmoc-EEA-OH	4.1±0.4	82±7	50±5
Fmoc-EEEA-OH	3.8±0.3	24±3	160±20
Fmoc-EEEEA-OH	3.9±0.4	5.9±0.4	660±50
Fmoc-EEEEEA-OH	3.7±0.3	1.0±0.1	3700±300
Fmoc-EEEEEEA-OH	3.5±0.3	1.0±0.1	3500±300

[a] The reaction was done in 0.05 M MES, pH 5.0, at 25°C for 2 min. k_{cat} and K_m values were measured at substrate concentrations ranging from 0.1 to 5.5 μM using 1.6 nM CPase Y. Only alanine was released under these conditions.

with CPase Y and Fmoc-peptide was formed by using the catalytically inactive mutant enzyme H397A[12] which shows the same binding efficiency for protein substrates as wild-type CPase Y.[5] Fluorescence of the complex was measured with the reaction mixture of 400 μl of 1.6 μM H397A in 50 mM MES buffer (pH 6.5) in the presence of 2.0 μM Fmoc-(Glu)$_{4,5}$Ala-OH using a RF-5300PC fluorophotometer (Shimadzu, Kyoto, Japan), excited at 280 nm.

Results

Kinetics of CPase Y-catalyzed hydrolysis of Fmoc-peptides

During a 2-min incubation, only C-terminal amino acid (alanine) was released with a linear increase from Fmoc-(Glu)$_n$Ala-OH to give an initial velocity of the reaction.

Table 2 shows kinetic parameters for CPase Y-catalyzed hydrolyses of Fmoc-(Glu)$_n$Ala-OH at pH 6.5 where γ-carboxyl groups of glutamic acid residues are in dissociated carboxylate anions. As the peptide length increases from n=1 to n=5, K_m values significantly decrease with only slight changes in k_{cat} values. Respective values of K_m and k_{cat} for Fmoc-(Glu)$_5$Ala-OH are almost the same as those for Fmoc-(Glu)$_6$Ala-OH. These values are also the same as those for protein substrates such as reduced- and carboxymethylated-ribonuclease A (RCM-RNase A) and RCM-lysozyme as described previously.[5] These results show that CPase Y has four subsites (S_2-S_5) in addition to widely accepted subsites S_1' and S_1.[1,3,4]

Table 4. Subsite Affinity, A_n, of CPase Y for Glutamate and Glutamic Acid

Subsite	A_n for glutamate (kJmol^{-1})	A_n for glutamic acid (kJmol^{-1})	ΔA_n (kJmol^{-1})
S_2	4.4	2.7	1.7
S_3	3.4	3.0	0.4
S_4	3.2	3.4	−0.2
S_5	7.3	4.4	2.9
S_6	0.20	0.0	0.2

K_m for Fmoc-(Glu)$_n$Ala-OH was measured at pH 6.5 for glutamate and at pH 5.0 for glutamic acid, and A_n and ΔA_n were calculated by the following equations;

$$A_n = RT\ln \frac{K_m \text{ for Fmoc-(Glu)}_{n-1}\text{Ala-OH}}{K_m \text{ for Fmoc-(Glu)}_n\text{Ala-OH}}$$

and $\Delta A_n = A_n$ for glutamate $- A_n$ for glutamic acid

Table 3 shows kinetic parameters for CPase Y-catalyzed hydrolyses of Fmoc-(Glu)$_n$Ala-OH at pH 5.0 where γ-carboxyl groups of glutamic acid residues are assumed to be in undissociated forms. These kinetic parameters reflect the CPase Y-preference for undissociated glutamic acid residues but not carboxylate anions, since K_m at pH 5.0 were much smaller than those at pH 6.5, and k_{cat} at pH 5.0 were slightly larger than those at pH 6.5. Different values of K_m and k_{cat} between pH 6.5 and 5.0 have been shown previously with acylated dipeptides as substrates.[10] These results show that subsites S_1-S_5 prefer uncharged or hydrophobic side-chains of substrates over negatively charged one.

Table 4 shows subsite affinity of CPase Y calculated from equation 1 and the difference of A_n values

(ΔA_n) between glutamate and glutamic acid from equation 2. Subsite affinities, $A_{2\text{-}5}$, are positive but affinity of S_6 is nearly zero. Positive A_n values mean that there is an interaction between substrate and corresponding subsite in spite of dissociated and undissociated forms of γ-carboxyl group of glutamic acid residues. Nearly zero A_6 values at pH 6.5 and 5.0, which correspond to the affinity of subsite S_6, show that subsite S_6 of CPase Y does not interact with the substrate.

Prediction of Subsite S_5

The property of S_5 subsite was predicted from the fluorescence experiment. The maximum wavelength of intrinsic fluorescence of CPase Y was red-shifted and its intensity was decreased in the presence of Fmoc-$(Glu)_5$Ala-OH, but intrinsic fluorescence was not influenced by the presence of Fmoc-$(Glu)_4$Ala-OH (Fig. 1). These results show Glu residue at P_5 position influences the environment of some tryptophan residue in CPase Y. Therefore it is appropriate that CPase Y has a tryptophan residue at the S_5 subsite.

Kinetics of CPase Y-catalyzed hydrolysis of Fmoc-peptide amides

In CPase Y-catalyzed hydrolysis of Fmoc-$(Glu)_n$Ala-NH_2 (n = 0–5) at 25°C and pH 6.5 for 5 min, ammonia was released from Cbz-Ala-NH_2, Fmoc-$(Glu)_3$Ala-NH_2, and Fmoc-$(Glu)_5$Ala-NH_2, and an alanine amide was released from Fmoc-$(Glu)_1$Ala-NH_2, Fmoc-$(Glu)_2$Ala-NH_2, Fmoc-$(Glu)_4$Ala-NH_2, and Fmoc-Lys$(Glu)_3$Ala-NH_2 (Fig. 2 for example). Therefore, CPase Y acts as amidase or carboxamide peptidase depending on properties of peptide (Fig. 3).

Discussion

The dependence of K_m on Fmoc-$(Glu)_n$Ala-OH (n = 1–6) shows that CPase Y has six subsites (S_1' and S_1-S_5). This is consistent with the previous results with the dependence of k_{cat}/K_m on an oligopeptide substrate that CPase Y has six subsites.[23] In CPase Y, binding energy between side-chain of substrate and corresponding subsite is used only for the decrease of K_m.

In contrast to CPase Y, metallo-CPases, pancreatic CPases A and B, have five and six subsites, respectively.[14,15] However, their K_m for di- and oligopeptides are nearly equal,[15,16] showing that subsites S_2-S_4 of CPase A and S_2-S_5 of CPase B interact with corresponding position of substrates more weakly than subsites S_1' and S_1. This is due to the difference in the C-terminal carboxylate-binding mode between metallo-CPases and CPase Y. The former strongly binds to C-terminal carboxylate ion of the substrate via salt linkage with the side chain of Arg145 which functions as primary subsite,[14,17] and the latter enzyme

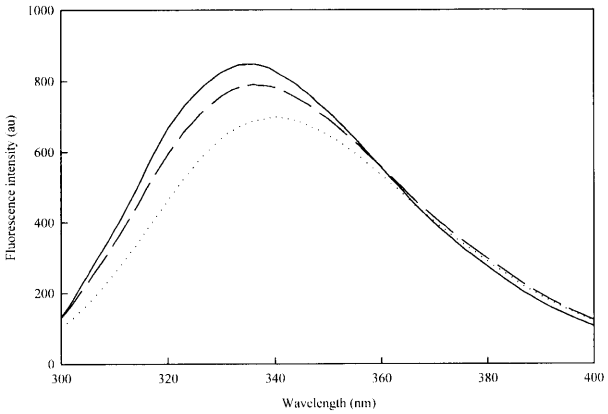

Fig. 1. Intrinsic Fluorescence Changes of H397A in the Presence and Absence of Fmoc-$(Glu)_{4,5}$Ala-OH.

Fifty micrograms of H397A CPase Y was suspended in 0.5 ml of 50 mM MES buffer (pH 6.5) in the presence and absence of 2.0 μM Fmoc-$(Glu)_{4,5}$Ala-OH and fluorescence spectra (em. 300–400 nm) with excitation at 280 nm were measured at 25°C. Solid line, H397A CPase Y; dashed line, + Fmoc-$(Glu)_4$Ala-OH; dotted line, + Fmoc-$(Glu)_5$Ala-OH.

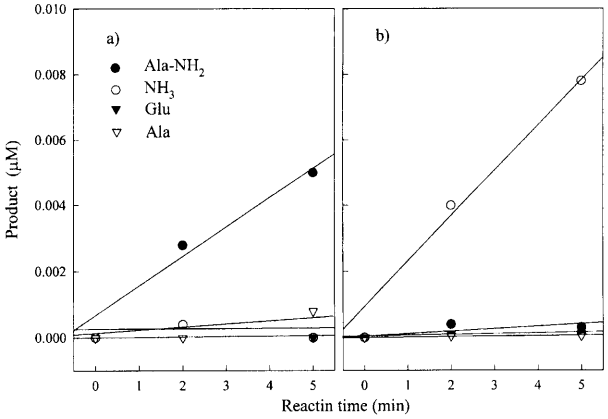

Fig. 2. Release of Amino Acid Amide and Ammonia by CPase Y-Catalyzed Hydrolysis of (a) Fmoc-$(Glu)_4$Ala-NH_2 and Fmoc-$(Glu)_5$Ala-NH_2 at 25°C and pH 6.5.

The released ammonia or Ala-NH_2 was measured by an amino acid analyzer. The reaction was done at 10 μM substrate concentration using 0.8 nM CPase Y in 0.05 M MES, pH 6.5, at 25°C for 5 min.

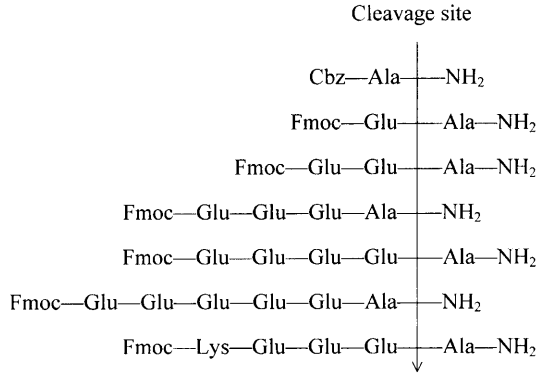

Fig. 3. Cleavage Sites of Fmoc-$(Glu)_n$Ala-NH_2 (n = 1-5) and Fmoc-Lys$(Glu)_3$Ala-NH_2 by CPase Y.

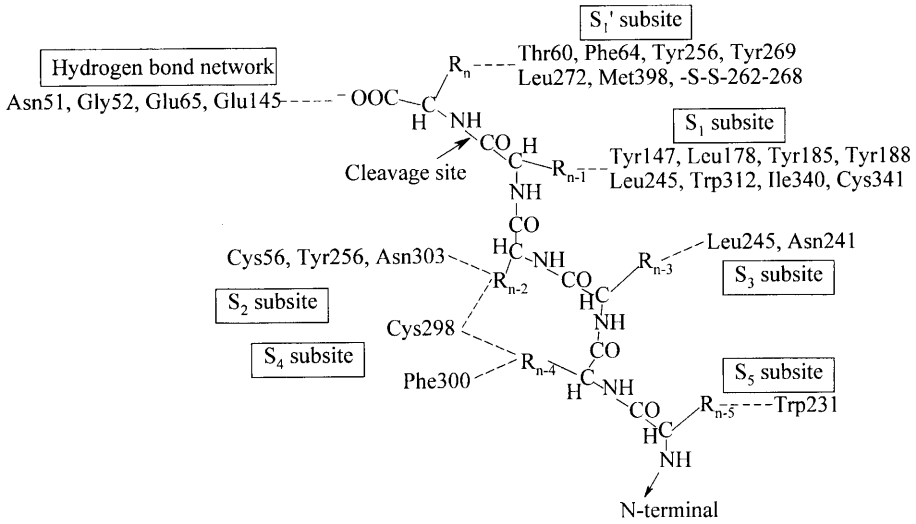

Fig. 4. Interaction between Substrate and Subsites in CPase Y.

rather weakly binds to the C-terminal carboxylate via a hydrogen bond network which is composed of an amide backbone of Gly52 and side chains of Asn51 and Glu145.[2] Therefore, CPase Y needs additional strong subsites in order to bind the substrate firmly to compensate for weak recognition of C-terminal carboxylate of the substrate.

ΔA_n values give us the information about corresponding subsite properties. Small ΔA_3 and ΔA_4 values of subsites S_3 and S_4 indicate that subsites S_3 and S_4 consist of weak positive or hydrophilic amino acid residues. On the other hand, large positive ΔA_2 and ΔA_5 values suggest that S_2 and S_5 subsites consist of positively charged or hydrophilic amino acid residues.

Wheat CPase WII (CPase WII) is classified in the serine CPase group and homologous to CPase Y with respect to structure and function. Crystallographic study of the complex with chymostatin indicates that the enzyme has five subsites, S_1' and S_1-S_4.[18] This enzyme also recognizes the C-terminal carboxylate of substrates by a hydrogen bond network.[19]

Overall structures of CPases Y and WII are very similar in spite of having different amino acid sequences.[1,18] Structural comparison of a complex of CPase WII and chymostatin with CPase Y allows us to propose the amino acid residues participating in subsites S_2-S_4 of CPase Y. Residues that participate in hydrogen bond network and subsite S_1' and S_1 have been shown from kinetic and crystallographic studies.[1-4] Figure 4 shows predicted interactions between substrate and subsites of CPase Y. Subsite S_2 of CPase Y is constructed with residues Cys56, Cys298, Tyr256, and Asn303, because of the resemblance to CPase WII where the side chain of leucine residue at P_2 position of chymostatin interacts with disulfide bridge between Cys56 and Cys303, Tyr239, and Asn306. These positions correspond to in CPase Y, respectively.[1,24] Subsite S_3 of CPase Y may be composed of residues Asn241 and Leu245 because the side chain of cyclic arginine residue at P_3 position of chymostatin interacts with corresponding Phe215 and Ile216 in CPase WII. Subsite S_4 of CPase Y may be composed of Cys298 and Phe300 because the side chain of the phenylalanine residue at P_4 position of chymostatin interacts with corresponding Cys303 and Asn303b. The N-terminal residue of chymostatin seems to interact with Trp231 of CPase Y, which is likely to construct subsite S_5. This proposal is supported by the fluorescence quenching with Fmoc-$(Glu)_5$Ala-OH, as shown in Fig. 1. It is suggested that Trp231 forms a hydrophobic patch on the protein surface together with other surrounding residues and this patch may be involved in substrate recognition.[1] This is the first result showing that Trp231 interacts with the substrate. These residues of subsite S_2-S_5 are consistent with the properties shown by the ΔA_n values. The position of subsite S_5 implies the P_n ($n > 6$) amino acids do not interact with the CPase Y molecule. This is a reason that CPase Y does not have more than an S_5 subsite. The antipain complex of CPase WII has the same conformation as the chymostatin complex.[18] Taking this into the consideration, these subsites proposed above seem to be involved in all cases of substrate binding of CPase Y.

It is of interest how CPase Y chooses the priority whether it acts as an amidase or carboxamide peptidase for amide substrates. It is suggested that the amidase activity of CPase Y is lower than the carboxamide peptidase activity due to the lack of P_1'-S_1' interactions which use a hydrogen bond network.[20] The recognition mechanism of the C-terminal amide group in case of carboxamide peptidase reaction has been proposed as follows[11]: Asn51 donates a hydrogen bond to the C=O group of the C-terminus of the substrate, and Glu145 (in the charged form) accepts

one from the NH_2 group of the substrate. However, this recognition mechanism may be quite weak. It is likely that sum of interactions between the side chains of peptides and corresponding subsites compensate the lack of P_1'-S_1' interaction, so the amidase activity prevails for Fmoc-$(Glu)_{3,5}$Ala-NH_2. This is probably a reason why the priority between amidase and carboxamide peptidase activities depends on the substrate.

It is a question why the carboxamide peptidase activity prevails for Fmoc-$(Glu)_4$Ala-NH_2. We assume that the S_5 subsite may have negative charges, so a negative charge of the glutamate residue at the N-terminus of Fmoc-$(Glu)_4$Ala-NH_2 is a disadvantage for an effective interaction with S_5 subsite. However, this assumption is eliminated because CPase Y showed carboxamide peptidase activity for Fmoc-Lys$(Glu)_3$Ala-NH_2 (Fig. 3). This result suggests that there is no negative charge in the S_5 subsite, and this suggestion is consistent with fluorescence experiments described above. However, further studies are needed to discover the determinant of the priority between amidase and carboxamide peptidase activities.

Peptide amides are not substrates for CPases A and B.[14,21,22] This is because the amide part of substrates is not recognized by Arg145 of CPases A and B. Further substrate binding with other sites are too weak to compensate for the lack of salt linkage.

In contrast, CPase Y acts as a degrading enzyme for several unneeded proteins, peptides, and peptide amides in the vacuole of *Saccharomyces cerevisiae*. This diversity of the C-terminus of substrates may make the C-terminal region recognition advantageous to CPase Y. CPase Y uses hydrogen bonds to recognize C-terminal group,[2,11] while CPase A uses a salt linkage between substrate carboxyl group and Arg145.[14] To compensate for the weak hydrogen bonding, CPase Y requires multi-subsites, which result in high and homogeneous affinity to a variety of substrates.

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