**Prediction of the Mechanism of Action of Omuralide (*clasto*-Lactacystin
 β -Lactone) on Human Cathepsin A Based on a Structural Model of the Yeast
Proteasome β 5/PRE2-Subunit/Omuralide Complex**

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Abstract

Cathepsin A (CathA) is a lysosomal serine carboxypeptidase that exhibits homology and structural similarity to the yeast and wheat serine carboxypeptidases (CPY and CPW) belonging to the α/β -hydrolase fold family. Human CathA (hCathA) and CPW have been demonstrated to be inhibited by a proteasome (threonine protease) inhibitor, lactacystin, and its active derivative, omuralide (*clasto*-lactacystin β -lactone), as well as chymostatin. A hCathA/omuralide complex model constructed on the basis of the X-ray crystal structures of the CPW/chymostatin complex and the yeast proteasome β -subunit (β 5/PRE2)/omuralide one predicted that the conformation of omuralide in the active-site cleft of proteasome β 5/PRE2 should be very similar to that of chymostatin at the S1 catalytic subsites in the hCathA- and CPW-complexes. The relative positions of the glycine residues, *i.e.*, Gly57 in hCathA, Gly53 in CPW, and Gly47 in β 5/PRE2, present in the oxyanion hole of each enzyme were also highly conserved. These results suggest that omuralide might inhibit hCathA and CPW at the S1 subsite in their active-site clefts through direct binding to the active serine residue.

1. Introduction

Cathepsin A (CathA; EC3.4.16.1) is a mammalian enzyme that exhibits serine carboxypeptidase activity at acidic pH and deamidase/esterase activities at neutral pH toward artificial substrates and a set of bioactive peptides, respectively [1-3]. It exhibits amino acid sequence homology and structural similarity to the yeast and wheat serine carboxypeptidases (CPY and CPW), which belong to the so-called α/β hydrolase fold family [4,5]. The enzyme is also known as lysosomal protective protein/cathepsin A (PPCA), which has a protective function as to lysosomal glycosidases including neuraminidase 1 (NEU1, EC3.2.1.18) and β -galactosidase (β -Gal, EC3.2.1.23), forming a multienzymic complex in lysosomes to activate the former enzyme and to stabilize the latter one [4,6,7]. Human PPCA is synthesized as a 54-kDa inactive precursor/zymogen and then proteolytically processed to the active mature 32/20-kDa two-chain form [4,6]. The mature form (CathA) is also secreted from human platelets and lymphoid cells [1,8], and exhibits extracellular functions *in vivo*.

Galactosialidosis is an autosomal recessive lysosomal storage disease caused by a primary defect of the PPCA gene, located on chromosome 20q13.1, associated with simultaneous loss of protective and catalytic functions, and excessive accumulation of natural substrates in tissues and urine [6,9]. Most patients develop loss of vision as an initial symptom at the age of 10~15 years, followed by neurosomatic manifestations, including cerebellar ataxia, action myoclonus, cherry-red spots, coarse facies, angiokeratoma, and viscerosplenomegaly. PPCA gene-disrupted mice have been utilized as a human disease model for elucidating the pathogenic mechanism and for evaluating therapeutic approaches [10-12]. However, the pathophysiological effect of loss of the

catalytic function could not be discriminated from that of the defect of its protective function. Accordingly, the pathophysiological role of the catalytic function of CathA remains to be elucidated, although we demonstrated that endothelin-1, as a putative natural substrate of CathA, is distributed abnormally in the brains from aborted galactosialidosis patients [13]. If a specific inhibitor of CathA activity could be developed that does not affect the protective function, it would be a very useful tool for analysis of the pathogenic role of the catalytic function of CathA.

Serine carboxypeptidases are widely distributed in eukaryotes and are classified into two types; C-type carboxypeptidases (EC3.4.16.5) with a preference for hydrophobic amino acid residues at the P1' position, and D-type ones (EC 3.4.16.6) exhibiting high affinity for positively charged (basic) P1' residues [14,15]. All these exopeptidases have a Ser-Asp-His "catalytic triad" in their active sites, and are inactivated by specific serine protease inhibitors including diisopropyl fluorophosphates (DFPs). Among them, yeast carboxypeptidase Y (CPY) [15] and wheat serine carboxypeptidase II (CPW) [16] are typical C- and D-type enzymes, respectively. Human CathA preferentially hydrolyzes N-blocked artificial dipeptide substrates having positively charged and hydrophobic amino acid residues at the P1' position at acidic and neutral pH [1,17]. The enzyme activity is also strongly inhibited by DFPS and mercurial compounds reacting with a free SH group in the active site [1,2]. These homologous serine carboxypeptidases exhibit structural similarity, especially in their active-site clefts [5]. However, these enzymes also exhibit differences in substrate specificity and sensitivity to inhibitors probably due to the presence of some specific amino acid residues in their active sites [17]. Recently, we analyzed the effects of microbial serine carboxypeptidase inhibitors, including chymostatin, pepstatin A, ebelactone B, lactacystin and omuralide, on

homologous human CathA, CPW and CPY, and demonstrated that they showed different sensitivities to the inhibitors under different pH conditions [18]. Interestingly, lactacystin and omuralide (active form of lactacystin with a β -lactone ring), well-known as potent inhibitors for cytoplasmic proteasomes [19,20], have also been revealed to have strong inhibitory effects on human CathA, CPW and CPY [18,21,22].

In this study we constructed docking models of them with omuralide on the basis of the X-ray crystal structures of the chymostatin-CPW complex and the yeast proteasome β -subunit (β 5/PRE2)/omuralide one. We also predicted that omuralide might inhibit these homologous serine carboxypeptidase activities through a very similar mechanism to that of chymostatin via covalent binding to the active serine residues in their active sites.

2. Materials and methods

2.1. Structural data for CPW, hCathA, CPY, and the yeast 20S proteasome β 5/PRE2 subunit

Structural data for CPW, hCathA and CPY were obtained from the Protein Data Bank (PDB) [23]. For this study, the structures of CPY (PDB code; 1CPY) [15], hCathA precursor (PDB code; 1IVY) [5], and the CPW/chymostatin-arginine complex (PDB code; 1BCS) [24] were used. As a putative mature form of hCath, we used the structure model obtained by removing the maturation subdomain from the precursor form, because the active site cleft in the precursor form of hCathA is simply blocked by the maturation subdomain (residues Ala254-Pro302) [5]. The yeast 20S proteasome

β 5/PRE2 subunit/omuralide complex structure was kindly provided by Drs. Groll and Huber [25].

2.2. Construction of hCathA/chymostatin-arginine and CPY/chymostatin-arginine complex models

As mentioned in the introduction, the serine carboxypeptidases used in this study each have the same Ser-Asp-His “catalytic triad” in the active site. To construct complex models, the structures of hCathA and CPY were superimposed on that of the CPW/chymostatin-arginine complex based on the C α atom coordinates of the catalytic residues by means of the least-square-mean fitting method [26,27], and chymostatin-arginine was docked into each structure. Then, the sites of interaction between chymostatin and the superimposed hCathA and CPY structures were extracted based on the atom distance (< 4.0 [Å]). The docking models were built using molecular modeling software, SYBYL/BIOPOLYMER (TRIPOS, St. Louis, Mo.).

2.3. Construction of hCathA/omuralide, CPY/omuralide and CPW/omuralide complex models

The proteasome β 5/PRE2 subunit is known as a threonine protease and its catalytic residues are different from those of serine carboxypeptidases. However, the proteasome inhibitor omuralide also has inhibitory effects on serine carboxypeptidases, i.e., hCathA and CPW. Therefore, we tried to superimpose the CPW/chymostatin-arginine complex on the proteasome β 5/PRE2 subunit/omuralide complex based on the essential atom

positions for peptide hydrolysis; the nucleophile oxygen (O_γ at Ser146 in CPW and O_γ at Thr1 in $\beta 5/\text{PRE2}$), the proton acceptor nitrogen ($N\epsilon 2$ at His397 in CPW and N at Thr1 in $\beta 5/\text{PRE2}$), and the inhibitor's carbon corresponding to the hydrolysis target carbonyl carbon ($C9$ in chymostatin and $C4$ in omuralide). Thus, the CPW/chymostatin-arginine complex was firstly superimposed on the proteasome $\beta 5/\text{PRE2}$ subunit/omuralide complex based on the essential atom positions by means of the least-square-mean fitting method [26,27]. Next, hCathA and CPY were superimposed on the CPW/chymostatin-arginine complex in the same manner as previously described. Now that the structures of CPW, hCathA and CPY had been superimposed on the proteasome $\beta 5/\text{PRE2}$ subunit/omuralide complex, the omuralide was docked into each structure. Then, the sites of interaction between omuralide and the superimposed CPW, hCathA and CPY structures were extracted based on the atom distance (< 4.0 [Å]). The docking models were also built using molecular modeling software, SYBYL/BIOPOLYMER (TRIPOS).

3. Results

Structural models of serine carboxypeptidases and their chymostatin-arginine complexes

As shown in Fig. 1, we first built structural models of the monomeric forms of CPW (panels A and D), hCathA (panels B and E), and CPY (panels C and F). In particular, the hCathA model was constructed on the basis of the X-ray crystal structure of the PPCA precursor by deleting the submaturation subdomain (residues 254-302) in the cap

domain (residues 183-302), including the blocking peptide (residues Asp272-Phe277) and the excision peptide (residues Met285-Arg298), to predict its enzymatically active mature form. As shown in the lower panels (D to F) of Fig. 1, significant conformational changes of the active-site clefts located in the core domains of the three enzymes were hardly predicted to occur among these models. The orientations of the side chains of these conserved amino acid residues located in the active sites, including the Ser-Asp-His catalytic triad, oxyanion hole and free cysteine residues of these serine carboxypeptidases, were quite similar with the exception of that the free cysteine residues in hCathA and CPY (Cys375 and Cys341, respectively) were replaced by the Val341 residue present in CPW.

Fig. 1 shows the complex models of hCathA (panels B, H and K) and CPY (panels C, I and L) with chymostatin and a putative reaction product, arginine, obtained by utilizing the X-ray atomic coordinates of the ternary complex of CPW with chymostatin and arginine (panels A, G and J). Basically, the locations of the docked chymostatin and arginine in the active-site clefts of hCathA (panel H) and CPY (panel I) were predicted to be very similar to those in the case of the CPW complex (panel G). Table I summarizes the interactions between the active site residues of the enzymes and chymostatin at P1 to P4. Common residues were predicted to be conserved in CPW (Gly53, Cys56, Tyr147, Leu178, Tyr239, Cys303 and Asn306), the hCathA model (Gly57, Cys60, Tyr151, Leu180, Tyr247, Cys334 and Asn339), and CPY (Gly53, Cys56, Tyr147, Leu178, Tyr256, Cys298 and Asn303). However, there were still differences in amino acid residues among the enzymes (CPW, Phe215, Ile216 and Asp303 in panel J; hCathA, Arg344 and Phe336 in panel K; and CPY, Trp312 and Phe300 in panel L). In particular, the cyclic arginine residue at the P3 position of chymostatin was suggested to

cause electrostatic repulsion of Arg344 in the S3 subsite of hCathA because of the presence of positively charged nitrogen atoms (panel K). On the other hand, the phenylalanine residue at the P1 position of chymostatin was predicted to collide with the Trp312 residue located in both the S1 and S3 subsites of CPY (panel I). A difference between the negatively charged Asp303 residue in the S4 subsite of CPW, and aromatic Phe336 and Phe300 at the corresponding positions in the S4 subsites of hCathA and CPY, respectively, was also observed in these models.

We also investigated structure of the yeast 20S proteasome $\beta 5$ /PRE2 subunit/omuralide complex (Fig. 2A) determined by the X-ray crystallography [24]. The location of the covalently bound omuralide and the amino acid residues, below 4.0Å apart, possibly interacting with the inhibitor in the active site of the $\beta 5$ /PRE2 subunit via hydrogen bonds and hydrophobic interactions is indicated in Fig. 2B. In particular, the covalent binding of the O γ atom of the active Thr1 residue to the C4 atom of omuralide, and a significant interaction between the O and N atoms in the main chain of the Gly47 residue and the imino and carbonyl groups of omuralide, respectively were observed.

Because of the similar interactions between the O γ atom of the active Ser146 residue and the C9 atom of chymostatin as well as between the Gly53 residue in the oxyanion hole of CPW and P1 of chymostatin in the complex structure, we examined the superimpositioning of chymostatin in the complex with CPW on omuralide in that with the proteasome $\beta 5$ /PRE2 subunit (Fig. 3). Interestingly, the O γ atom of Ser146 and N ϵ 2 of His397, composing a Ser-Asp-His catalytic triad of CPW, and the C9 atom of chymostatin were predicted to be superimposable on the O γ and N atoms of Thr1 in the $\beta 5$ /PRE2 subunit and the C4 atom in omuralide, respectively, when the C11 and C12

atoms of omuralide are located at the position corresponding to P1 of chymostatin, as shown in Fig. 3B and E. The superimpositioning of Gly53 in CPW on Gly47 in the β 5/PRE2 subunit could also be observed. The results suggest that the relative positions of nitrogen and oxygen atoms in their active sites and the glycine residues present in the oxyanion hole of each enzyme are very similar in the two complex structures. The common interactions between the inhibitors and the enzymes are depicted in Fig. 3F.

We further constructed a hCathA/omuralide complex model (Fig. 4A) based on the structural model for the CPW/omuralide and β 5/PRE2 subunit/omuralide complexes (Fig. 4B and C). Omuralide could be docked well in the active-site cleft of hCathA, as expected from the quite similar structures of CPW and hCathA. As summarized in Table II, the common amino acid residues possibly interacting with omuralide in the complex models were Gly52, Gly53, Ser146, Tyr147, Leu178 and His397 in CPW (Fig. 4E), and Gly56, Gly57, Ser150, Tyr151, Leu180 and His429 in hCathA (Fig. 4D). The distances between the atom pairs were below 4.0Å in these models. In addition, the Val340 residue in CPW (Fig. 4E) was also predicted to possibly interact with omuralide, while the distances between the atom pairs of omuralide and the Ala374 in hCathA (Fig. 4D) corresponding to the Val340 in CPW were over 4.0Å. The results are summarized in Table III. The complex models exhibited the difference in space around the C6, C7, C13 and O9 atoms of omuralide of each peptidase. In particular, the hCathA/omuralide complex model suggested that molecular alteration at the atomic positions of C6, C7 and O9 might be effective for the designing of a more specific inhibitor for hCathA.

4. Discussion

Lactacystin and omuralide (*clasto*-lactacystin β -lactone) are well-known specific inhibitors of cytoplasmic multicatalytic proteasomes that exhibit three peptidase activities, *i.e.*, chymotrypsin-like, trypsin-like, and peptidylglutamylpeptidase activities [19,20]. The substances inhibit the chymotrypsin-like peptidase activity via covalent binding between the β -lactone rings of active derivatives, and the threonine residues of the β - and X-subunits of the yeast and mammalian proteasomes, which elicit many biological phenomena including the induction of neurite outgrowth from murine neuroblastoma cell line Neuro2a [28-30]. On the other hand, Ostrowska et al. reported that the CathA-like enzyme in human platelets was separable from proteasomes, and was also inhibited by lactacystin and omuralide [21,22], suggesting that these inhibitors are not necessarily specific for proteasomes, and that some of the biological effects of these inhibitors might be due to the inhibition of CathA activity. We also demonstrated directly that recombinant human CathA expressed in human fibroblastic cells with galactosialidosis as well as homologous CPW and CPY could be inactivated by treatment with these inhibitors, which suggested that they might exhibit a non-competitive and irreversible mode of inhibition for hCathA activity [18]. In contrast, another inhibitor, Z-Ile-Glu-(*O*-*t*-butyl)-Ala-leucinal (PSI), specific for proteasomes [30], did not show any inhibitory effect on the CathA activity (data not shown).

In this study we attempted to predict the inhibition mechanism of the active form of lactacystin (omuralide) with the β -lactone moiety for hCathA by structural modeling based on the X-ray crystal structures of homologous peptidases and their inhibitor-enzyme complexes.

First, we constructed monomeric active form models of serine carboxypeptidases on the basis of the X-ray atomic coordinates of each enzyme, hCathA precursor (PDB

code; 1IVY) [5], CPW (3SC2) [16], and CPY (1CPY) [15]. The conformation of the conserved amino acid residues in the active sites, including the Ser-Asp-His catalytic triad and oxyanion hole, were predicted to be very similar in the present models. The hCathA monomer model was constructed by removing the structure corresponding to the total maturation subdomain (residues 254-302) including the blocking peptide from the hCathA precursor molecule, on the premise that the conformation of the active sites of hCathA should be retained because of the existence of the disulfide bond between the Cys253 and Cys303 residues.

Previously we demonstrated that lactacystin and omuralide exhibit potent inhibitory activity toward each carboxypeptidase [18]. Omuralide had the IC₅₀ value 0.099 μ M at pH 5.6 for hCathA while the value for 20S proteasome β 5/PRE2 subunit was 0.10 μ M at pH 7.5. These findings suggest that there should be a common underlying inhibition mechanism, probably due to the covalent and irreversible binding of omuralide to the active serine residues in the catalytic triad. Another possibility is that the inhibitor may abolish CathA activity through binding to the free cysteine residue located in the active site because CPY and hCathA have been reported to have a free cysteine residue that is inactivated to react with heavy metal ions and iodoacetamide [1,3,15]. However, in our previous comparative analysis omuralide was also found to have an efficient, inhibitory effect on CPW [18], which has no free cysteine residue in its active site, as shown in Fig. 1D. In addition, the distance between atom pairs of omuralide and S γ atom in the side chain of free Cys375 was above 4.3 $\text{\AA}$ in hCathA/omuralide docking model. Moreover, there is little space for omuralide to bind directly with Cys375. Because, the S γ atom of Cys375 is located at the bottom of active-site cleft in hCathA (Fig.1E) and is close to surrounding atoms; the main chain O atom of Asn178 (3.2 $\text{\AA}$), the main chain O atom of

Ala374 (3.5 Å), the side chain Cε1 atom of His429 (3.6 Å) and the main chain N atom of Leu180 (3.7 Å). Therefore, it was suggested that omuralide could inhibit serine carboxypeptidases via direct interaction with an active serine residue but not necessarily with the free cysteine residues in their active sites.

Chymostatin is a potent inhibitor of chymotrypsin [31], but has an inhibitory effect on CPW activity through the formation of a covalent adduct with the active serine residue [23]. We also revealed that chymostatin has a relatively low inhibitory effect on hCathA as compared to that on CPW, and hardly inhibits CPY activity [18]. As X-ray crystal data for the CPW/chymostatin-arginine complex (PDB code, 1BCS) [24] are available, we further built hCathA- and CPY/chymostatin-arginine complex models on the structural basis of 1BCS. In that study all four residues (P1-P4) of chymostatin fitted well to the S1 through S4 subsites of the CPW structure, and the aldehyde group formed a covalent adduct with the active Ser146 residue of CPW. In the S1 subsite, the Val340, Val341, Phe215 and Ile216 residues were shown to form a hydrophobic binding surface for the P1 phenylalanyl side-chain of the compound, but still to accept positively charged amino acid residues [17]. In particular, the cyclic arginine residue at the P3 position of chymostatin was shown to be stabilized in the S3 subsite via interaction with the oxygen atoms of Phe215 and Ile216. The docking models revealed that the P1 through P4 residues of chymostatin could be docked in the active-site cleft in the hCathA model if the backbone of the inhibitor could form hydrogen bonds with the conserved Gly57, Tyr151, Tyr247 and Asn339 residues. However, the putative positive charge of the cyclic arginine residue of the docked chymostatin was predicted to be electrostatically repulsed by that of the Arg344 residue in the S3 subsite. This was considered to be another reason why chymostatin did not have a potent inhibitory effect

on hCathA activity. In the CPY complex model the P1 residue was predicted to collide with the Trp312 residue located in both the S1 and S3 subsites, suggesting why chymostatin had little effect on CPY activity. Thus, the structural factors due to the nonconserved amino acid residues in the active site clefts of serine carboxypeptidases may explain the difference in sensitivity of the homologous serine carboxypeptidases to chymostatin reported previously [18]. Recently, we also predicted electrostatic repulsion between the P3 cyclic arginine residue and the Arg344 residue of hCathA on molecular calculation of the interaction energy (Yoshida, submitted).

Next we investigated a proteasome $\beta 5$ /PRE2 subunit/omuralide complex structure determined by the X-ray crystallography and compared the conformation of omuralide in the $\beta 5$ subunit complex to that of chymostatin in CPW. As a result, conformational similarity of the inhibitors in the complex structures was predicted because the O_γ atom of Ser146 and $N\epsilon 2$ of His397, comprising the Ser-Asp-His catalytic triad of CPW, and the C9 atom of chymostatin could be superimposed on the O_γ and N atoms of Thr1 in the $\beta 5$ /PRE2 subunit and the C4 atom in omuralide, respectively, when the C11 and C12 atoms of omuralide are located at the position corresponding to P1 of chymostatin (see Fig. 3).

We further built CPW- and hCathA/omuralide complex models to predict the inhibition mechanism of omuralide on these serine carboxypeptidases by utilizing the conformational similarity between omuralide and chymostatin in the active sites of the $\beta 5$ /PRE2 subunit and CPW, respectively. In these models, a set of atoms, i.e., O_γ at Ser150 and $N\epsilon 2$ at His429 in hCathA, as well as O_γ at Ser146 and $N\epsilon 2$ at His397 in CPW, and C9 in chymostatin, could be superimposed on O_γ and N of Thr1 in $\beta 5$ /PRE2 and C4 in omuralide, respectively. The relative positions of the glycine residues, i.e.,

Gly57 in hCathA, Gly53 in CPW, and Gly47 in the β 5/PRE2 subunit, present in the oxyanion hole of each enzyme, were also highly conserved. These results suggest that omuralide might inhibit hCathA and CPW at the S1 subsite in their active-site clefts through direct binding to the active serine residue.

On the other hand, differences in the space around the C6, C7, C13 and O9 atoms of omuralide were observed in the docking models. As shown in Fig.2B, there is little space for other functional group to exist around omuralide in 20S proteasome β 5/PRE2 subunit/omuralide complex, especially near the O9 atom of omuralide because the N in the main chain of Thr21 (3.2 Å) as well as the C α of Ala20 (3.3 Å) are adjacent to the O9. In contrast, the nearest O atom in the main chain of Gly57 is at least 4.6 Å apart from the O9 of omuralide in hCathA/omuralide docking model. By taking this local space into account, especially that at the C6, C7 and O9 positions of omuralide, it could be possible to design a more specific inhibitor for hCathA based on the molecular form of omuralide by modifying the corresponding atoms or functional groups to others, that could not enter the catalytic site of β 5/PRE2 subunit but effectively bind to that in hCathA.

As the pathophysiological role of the CathA activity *in vivo* has not been fully elucidated, it is necessary to develop a specific inhibitor for CathA as a probe. On the other hand, PPCA gene knock-out mice have been utilized as a human galactosialidosis model for elucidating the pathogenic mechanism and for developing therapeutic methods [10-12]. However, as the protective function as to glycosidases as well as the catalytic function were disrupted in the model mice, the pathophysiological effects due to the catalytic defect of PPCA were indistinguishable from those due to the defect of its protective function. Therefore, it is worthwhile discovering and developing a more specific and potent inhibitor for CathA activity to analyze the pathophysiological roles

of CathA in various experimental systems including human cultured cell lines. Shah et al. reported that the synthetic analogue of omuralide, PS-519 (C7 *n*-propyl analogue of *clasto*-lactacystin β -lactone) had been currently under clinical evaluation in the treatment of acute stroke and myocardial infarction [32]. In hCathA/omuralide docking model, the methyl group (C13) located in C7 of *clasto*-lactacystin β -lactone is exposed on the surface of the complex. PS-519 is a omuralide derivative, in which the methyl group (C13) is substituted to *n*-propyl one. As the *n*-propyl group of PS-519 is also expected to exist on the surface, PS-519 should be predicted to bind to the active-site cleft of hCathA and have its inhibitory effect on it. The accumulation of structural information concerning the homologous serine carboxypeptidase family is expected to facilitate the development of specific inhibitors for human CathA.

In conclusion, specific and potent inhibitors for hCathA should be developed on the basis of the predicted hCathA/omuralide docking model by taking the space around the C6, C7, C13 and O9 atom of omuralide into account, which are not available in 20S proteasome β 5/PRE2 subunit/omuralide complex.

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Figure legends

Fig. 1. Modeled three-dimensional structures of the chymostatin-arginine complexes with CPW, hCathA and CPY.

The monomeric complex structures of CPW (panel A), hCathA (panel B) and CPY (panel C) are shown. The secondary structures of the enzymes are each shown as a tube and ribbon drawing. α -helix (magenta), β -strand (yellow), and loop (cyan), respectively. The side chains of the conserved amino acid residues in their active sites are shown as a ball and stick drawing. The chymostatin colored orange and the arginine colored green, as a putative substrate product, are indicated as a stick drawing. The carbon, oxygen, nitrogen and sulfur atoms are colored white, red, blue and yellow, respectively. The amino acid residues interacting with chymostatin are also shown as a space-filled drawing. In the second panels, the amino acid residues including the Ser-His-Asp catalytic triad, Gly residues in the oxyanion hole and free cysteine residues in hCathA (panel E) and CPY (panel F) corresponding to the Val341 in CPW (panel D) are shown as a ball and stick drawing. In the third panels the common amino acid residues interacting with chymostatin in the complex models with CPW (panel G), hCathA (panel H), and CPY (panel I) are shown as a space-filled drawing. In the lowest panels the different residues interacting with chymostatin in the complexes with CPW (panel J), hCathA (panel K), and CPY (panel L) are also indicated as a space-filled drawing.

Fig. 2. Structure of the yeast proteasome 20S β 5/PRE2 subunit / omuralide complex.

In panel A omuralide colored orange and the amino acid residue possibly interacting the

subunit are depicted as a ball and stick drawing. The side chains of the amino acid residues in the active site (panel B) are colored gray, and the oxygen, nitrogen and sulfur atoms are colored red, blue and yellow, respectively.

Fig. 3. Conformation of inhibitors in the active-site clefts of complex models.

Chymostatin colored orange, omuralide colored green and the amino acid residues possibly interacting with the subunit are depicted as a stick drawing in panel A and as a ball and stick drawing in panel C. The side chains of the amino acid residues in the active site are colored gray, and the oxygen, nitrogen and sulfur atoms are colored red, blue and yellow, respectively. Chymostatin in the CPW complex (panel A) and omuralide in the $\beta 5$ /PRE2 subunit (panel C). The superimpositioning is depicted in panel B, where the C11 and C12 atoms of omuralide are located at the position corresponding to P1 of chymostatin. The structural formulae of chymostatin (panel D) and omuralide (panel E) are shown. The atomic positions of the chymostatin and omuralide molecules are numbered as described in the previous reports, [24] and [25], respectively. The common structures in chymostatin and omuralide interacting with the amino acid residues in the active sites are indicated in panel F. Hy indicates a hydrophobic amino acid residue.

Fig. 4. Comparison of the docking models of omuralide with peptidases.

The monomeric complex structures of hCathA (panel A), CPW (panel B) and the proteasome $\beta 5$ /PRE2 subunit (panel C) are shown. The secondary structures of the enzymes are each shown as a tube and ribbon drawing. α -helix (magenta), β -strand (yellow), and loop (cyan), respectively. Omuralide colored orange is indicated as a stick

drawing. The side chains of amino acid residues interacting with the inhibitor are shown as a ball and stick drawing. The carbon, oxygen, nitrogen and sulfur atoms are colored white, red, blue and yellow, respectively. In the lower panels the different amino acid residues interacting with omuralide, Val340 and Phe215 in CPW (panel E) are colored green, while Ala374 in hCathA (panel D) corresponding to the Val340 in CPW is colored cyan. The amino acid residues interacting with the omuralid in the $\beta 5$ /PRE2 subunit are also shown in panel F.

Figure 1. Aikawa et al.

Figure 2. Aikawa et al.

Figure 3. Aikawa et al.

Figure 4. Aikawa et al.

Table I.

Interaction between chymostatin and serine carboxypeptidases (CPW, hCathA and CPY).

Subsite	CPW	hCathA	CPY	Interaction (Atoms in chymostatin)
S1	Gly53	Gly57	Gly53	Hydrogen bond between Gly main chain NH and P1 CO(O1)
	Tyr147	Tyr151	Tyr147	Hydrogen bond between Tyr main chain NH and P1 CO(O1)
	Gly53	Gly57	Gly53	Hydrogen bond between Gly main chain CO and P1 NH(N1)
	Leu178	Leu180	Leu178	Adjacent to P1 side chain
	Phe215	–	–	Adjacent to P1 side chain
S2	–	–	Trp312^a	Collision of Trp side chain with P1 side chain
	Tyr239	Tyr247	Tyr256	Hydrogen bond between Tyr side chain OH and P2 CO(O2)
	Cys56	Cys60	Cys56	Adjacent to P2 side chain
S3	Cys303	Cys334	Cys298	Adjacent to P2 side chain
	Asn306	Asn339	Asn303	Hydrogen bond between Asn side chain Nδ2 and P3 CO(O3)
	Asn306	Asn339	Asn303	Hydrogen bond between Asn side chain Oδ1 and P3 NH(N6)
	Phe215	–	–	Phe main chain CO O adjacent to P3 side chain N+(N4)
	Ile216	–	–	Ile main chain CO O adjacent to P3 side chain N+(N4)
	–	–	Asn241	Asn side chain Oδ1 adjacent to P3 side chain N+(N4),N(N5)
	–	Arg344^a	–	Arg side chain Nη2 adjacent to P3 side chain N+(N4)
S3–S4	–	–	Trp312^a	Collision of Trp side chain with P3 side chain
S4	Asn306	Asn339	Asn303	Hydrogen bond between Asn side chain Oδ1 with ureido bond NH(N7)
	Asp303B	Phe336	Phe300	Adjacent to P4 side chain
	Cys303	Cys334	Cys298	Adjacent to P4 side chain

^aThe bold letters indicate the amino acid residues in the active site clefts of the enzymes that could cause steric hindrance or electrostatic repulsion with chymostatin.

Table II. Amino acid residues possibly interacting with omuralide in the docking models.

CPW	hCathA
Gly52	Gly56
Gly53	Gly57
Ser146	Ser150
Tyr147	Tyr151
Leu178	Leu180
Phe215	-
Val340	Ala374
His397	His429

The amino acid residues shown are those within 4.0Å from any atom of omuralide.

The bold letters indicate the amino acid residues exceptionally over 4.0Å apart from the atoms of omuralide in the docking models.

Table III. Differences in distance between the atomic pairs in serine carboxypeptidase/omuralide complex models.

Model	Atomic pair	Distance [$\text{\AA}$]
CPW/Omuralide	Val340-C γ 1---O9-Omuralide	3.8
	Val340-C γ 1---C6-Omuralide	3.4
	Val340-C γ 1---C7-Omuralide	4.0
	Val340-C γ 1---C13-Omuralide	4.7
hCathA/Omuralide	Ala374-C β 1---O9-Omuralide	5.0
	Ala374-C β 1---C6-Omuralide	4.2
	Ala374-C β 1---C7-Omuralide	5.0
	Ala374-C β 1---C13-Omuralide	5.4