Unusual Extra Space at the Active Site and High Activity for Acetylated Hydroxyproline of Prolyl Aminopeptidase from *Serratia marcescens*

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The prolyl aminopeptidase complexes of Ala-TBODA [2-alanyl-5-*tert*-butyl-(1, 3, 4)-oxadiazole] and Sar-TBODA [2-sarcosyl-5-*tert*-butyl-(1, 3, 4)-oxadiazole] were analyzed by X-ray crystallography at 2.4 Å resolution. Frames of alanine and sarcosine residues were well superimposed on each other in the pyrrolidine ring of proline residue, suggesting that Ala and Sar are recognized as parts of this ring of proline residue by the presence of a hydrophobic proline pocket at the active site. Interestingly, there was an unusual extra space at the bottom of the hydrophobic pocket where proline residue is fixed in the prolyl aminopeptidase. Moreover, 4-acetyloxyproline-βNA (4-acetyloxyproline β-naphthylamide) was a better substrate than Pro-βNA. Computer docking simulation well supports the idea that the 4-acetyloxy group of the substrate fitted into that space. Alanine scanning mutagenesis of Phe139, Tyr149, Tyr150, Phe236, and Cys271, consisting of the hydrophobic pocket, revealed that all of these five residues are involved significantly in the formation of the hydrophobic proline pocket for the substrate. Tyr149 and Cys271 may be important for the extra space and may orient the acetyl derivative of hydroxyproline to a preferable position for hydrolysis. These findings imply that the efficient degradation of collagen fragment may be achieved through an acetylation process by the bacteria.

Prolyl aminopeptidase (proline iminopeptidase; EC 3.4.11.5) catalyzes the removal of N-terminal proline from the peptides. This enzyme was first identified from *Escherichia coli* by Sarid et al. (21) and was found in several bacteria thereafter. The enzyme genes have been cloned from *Aeromonas sobria* (13), *Bacillus coagulans* (14), *Flavobacterium meningosepticum* (11), *Hafnia alvei* (12), *Lactobacillus delbrueckii* subsp. *bulgaricus* (3), *Neisseria gonorrhoeae* (1), *Thermoplasma acidophilum* (22), *Xanthomonas campestris* pv. *citri* (2), and *Serratia marcescens* (10).

Most bacteria that produce prolyl aminopeptidase are pathogenic, including *S. marcescens*, a gram-negative bacterium that causes opportunistic disease in humans. The role of prolyl aminopeptidase seems to break down peptides into amino acids as a nutrient. Prolyl activity is necessary because other aminopeptidases cannot release proline residue efficiently. This is especially important for the ability of some pathogenic bacteria to degrade collagen, which has a large number of Gly-X-Y repeat sequences, where X is often proline and Y is often hydroxyproline. Complete digestion of proline-containing peptides derived from collagen requires prolyl aminopeptidase as well as other aminopeptidases. These enzymes of pathogenic bacteria act especially on collagen degradation. Since many *S. marcescens* strains are resistant to multiple antibiotics, they represent a growing public health problem. Recently, many patients have been infected by this bacterium in hospitals, and some of them have died. New types of antibacterial drugs different from the ordinary antibiotics for *S. marcescens* are desired. It would be helpful to interfere with the

growth of those bacteria causing opportunistic diseases by inhibiting the activity of prolyl aminopeptidase.

We have cloned the prolyl aminopeptidase gene from the *S. marcescens* gene (10) and solved its three-dimensional structure by X-ray crystallography. The enzyme is composed of α/β-hydrolase fold and helix domains (24). The catalytic mechanism of the enzyme was studied using substrates and inhibitors that each contained a prolyl, alanyl, sarcosyl, L-α-aminobutyryl, or norvalyl group (8, 9).

In this paper, we report new findings of high activity for acetylated hydroxyproline and an unusual space in the active site of the prolyl aminopeptidase.

MATERIALS AND METHODS

Materials. Restriction endonucleases and other modifying enzymes were purchased from Toyobo Biochemicals or New England Biolabs. Proline β-naphthylamide (Pro-βNA) and Fast Garnet GBC were from Sigma Chemical. The oligonucleotide primers for site-directed mutagenesis were synthesized by Amersham Biosciences. Mutan-Super Express Km kit was from Takara Shuzo. ALFexpress AutoCycle sequencing kit and other reagents for DNA sequencing were obtained from Amersham Biosciences. Polyethylene glycol with a mean molecular weight of 6,000 was from Nacalai Tesque. Ala-TBODA [2-alanyl-5-*tert*-butyl-(1, 3, 4)-oxadiazole] and Sar-TBODA [2-sarcosyl-5-*tert*-butyl-(1, 3, 4)-oxadiazole] were synthesized previously (8).

Bacterial strains, plasmids, and media. *E. coli* MV1184 [*ara* Δ(*lac-proAB*) *rspL* *thi*(φ80*lacZ*Δ*M15*) Δ(*srl-recA*)306::Tn10(Tet^r) F' (*traD36 proAB*⁺ *lacI*^q *lacZ*Δ*M15*)] was used for site-directed mutagenesis. *E. coli* DH1 (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) and DH5α [*supE44* Δ*lacU169*(φ80*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] were used as hosts for expression. Plasmids pUC19 and pKF19k (Takara) were used as vectors for expression and mutagenesis, respectively. Bacteria were grown in LB broth (1% tryptone, 1% NaCl, and 0.5% yeast extract) or nutrient medium (N broth [1% polypeptone, 1% meat extract, and 0.5% NaCl]). The concentrations of antibiotics used in this study were as follows: ampicillin, 100 μg/ml (LB broth) or 50 μg/ml (N broth); kanamycin, 50 μg/ml.

Construction of the mutant enzymes by site-directed mutagenesis. Mutations were introduced by a PCR-based method using the Mutan-Super Express Km kit

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FIG. 1. The ribbon and stick model of enzyme-inhibitor complexes. $|F_o| - |F_c|$ omit maps of the enzyme-Ala-TBODA complex (A) and the enzyme-Sar-TBODA complex (B) were calculated without the structure factor of inhibitor. Each inhibitor is shown in red. The electron density map is shown at the 2σ contour level. (C) Stereo drawing of superimposed enzyme-Pro-TBODA (orange), Ala-TBODA (green), and Sar-TBODA (blue) complexes. Panels A and B were produced by the POVscript⁺ and Raster3D programs, and panel C was drawn by the MOLSCRIPT and Raster3D programs.

(Takara) following the manufacturer's instructions. Template plasmids for PCR were constructed through the following steps. pSPAP-HE (10) was digested with EcoRI and PstI, and the 1.3-kb fragment containing the entire SPAP gene was subcloned into the same restriction sites of pKF19k (Takara) to construct pSPAP-M1. pSPAP-M2, which contained a 1.0-kb SphI-EcoRI fragment, was constructed similarly in pKF19k as a vector. pSPAP-M1 was used as a template for mutants of F150A, and F236A mutants were made with pSPAP-M2 as a template using primers CATTGGTATGCCAGGACGCGC and AACCACTACGCCACCATCTG, respectively. Each mutation was confirmed by sequencing analysis, using an ALFexpress DNA sequencer. DNA fragments containing individual mutations were prepared by digesting the respective plasmids with EcoRI and PstI (for pSPAP-M1) or EcoRI and EcoRV (for pSPAP-M2). These fragments were inserted into the same sites of pUC19 (for pSPAP-M1) or pSPAP-HE (for pSPAP-M2). The cloned plasmids were then used to transform *E. coli* DH1 or DH5 α for expression. Other mutants were reported previously (9).

Purification of the mutant enzymes. Mutants were purified by essentially the same procedure as that used for the wild-type enzyme. *E. coli* DH1 or DH5 α harboring individual mutant plasmids was aerobically cultured in 12 liters of N broth containing 50 mg/liter ampicillin at 37°C for 17 h using a jar fermenter. The harvested cells were resuspended in 20 mM Tris-HCl (pH 8.0) and disrupted with glass beads in a Dyno mill. The cell lysate was centrifuged to remove cell debris. To remove chromosomes and viscous materials, 2% protamine sulfate dropwise was added to the clear supernatant to give 15 mg per gram of wet cells. The mixture was kept for 15 min at 4°C and then centrifuged at 12,000 rpm for 30

min. The supernatant was fractionated with ammonium sulfate from 40 to 80% saturation. The precipitate was dissolved in 20 mM Tris-HCl buffer (pH 8.0) saturated with 35% ammonium sulfate. The clear solution was applied to a column of Toyopearl HW65C equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 35% saturation ammonium sulfate. The absorbed enzyme was eluted with a decreasing linear gradient of ammonium sulfate concentration from 35 to 0% saturation in 20 mM Tris-HCl buffer (pH 8.0).

Assay of mutant enzyme activity and kinetic studies. The activity of prolyl aminopeptidase from *S. marcescens* was assayed using Pro- β NA, hydroxyproline- β -naphthylamide (Hyp- β NA), and 4-acetyloxypoline- β -naphthylamide (AcHyp- β NA) as substrates.

The reaction mixture consisted of 0.8 ml of 20 mM Tris-HCl (pH 8.0), 0.1 ml of enzyme solution, and 0.1 ml of 3 mM Pro- β NA. After a 10-min incubation at 37°C (unless specifically indicated), the reaction was stopped by adding 1 ml of Fast Garnet GBC (1 mg/ml) solution containing 10% Triton X-100 in 1 M sodium acetate buffer (pH 4.0).

The absorbance at 550 nm was measured after a 20-min incubation using a Hitachi model 1100 spectrophotometer. One unit of activity was defined as the amount of the enzyme that released 1 μ mol of β NA per min under the above conditions.

To determine the K_m values, the concentration of substrate was varied. Lineweaver-Burk plots were used to calculate the K_m and apparent V_{max} . The enzyme concentration was estimated from $E_{1\%,280\text{ nm}} = 0.98$, and a molecular weight of 36,083 was used for k_{cat} calculation.

Synthesis of Hyp- β NA and AcHyp- β NA. Hyp- β NA was synthesized using the standard procedure in solution by condensation of Z-Hyp (benzyloxycarbonyl-4-hydroxypropyl) and β -naphthylamine with water-soluble carbodiimide, and then the Z group was removed by hydrogenation over palladium carbon. AcHyp- β NA was also synthesized in solution by condensation of Z-Hyp and β -naphthylamine with water-soluble carbodiimide. The Z group was removed by the HBr acid method.

Data for Hyp- β NA were as follows: ^1H NMR (300 MHz, CD_3OD) δ 1.14 (1H, d, J = 6.6 Hz), 1.98 (1H, ddd, J = 13.2, 8.7, 4.8 Hz), 2.26 (1H, m), 2.98 (1H, dt, J = 12.0 Hz, 1.5 Hz), 3.11 (1H, dd, J = 12.0, 3.9 Hz), 4.90 (1H, t, J = 9.0 Hz), 4.23 (1H, m), 7.36 ~ 7.47 (2H, m), 7.79 (1H, dd, J = 12.6, 2.1 Hz), 7.75 ~ 7.83 (3H, m), 8.21 (1H, s); MS (FAB, NBA) 257 ($M + \text{H}$) $^+$.

Data for AcHyp- β NA were as follows: ^1H NMR (300 MHz, CD_3OD) δ 2.13 (3H, s), 2.43 (1H, ddd, J = 14.4, 9.3, 4.5 Hz), 2.77 (1H, ddt, J = 14.4, 7.5, 1.5 Hz), 3.59 (1H, dt, J = 12.9 Hz, 1.5 Hz), 3.75 (1H, dd, J = 12.9, 4.5 Hz), 4.67 (1H, dd, J = 12.0, 7.5 Hz), 5.50 (1H, t, J = 4.5 Hz), 7.39 ~ 7.50 (2H, m), 7.61 (1H, dd, J = 15.0, 2.1 Hz), 7.70 ~ 7.86 (3H, m), 8.26 (1H, d, J = 1.8 Hz); MS (FAB, NBA) 299 ($M + \text{H}$) $^+$.

Preparation of crystals of enzyme-inhibitor complex. Crystals of the enzyme-inhibitor complexes were obtained using the soaking method. The wild-type crystals have been crystallized by the hanging-drop vapor diffusion method using 50 mg/ml protein solution, along with a reservoir solution of 200 mM Na acetate, 20% (wt/vol) polyethylene glycol 6000, and 100 mM Na cacodylate- KH_2PO_4 buffer, pH 6.5 (24). A droplet was composed of 3:2 (vol/vol) of protein solution and reservoir solution. The wild-type crystals were soaked in the prepared standard solution, which had the same composition as the reservoir solution but also contained 10 mM inhibitor (Sar-TBODA) for 7 h and 1 mM inhibitor (Ala-TBODA) for 7 days at 293 K. The crystals of enzyme-Sar-TBODA and enzyme-Ala-TBODA complexes belonged to space group $P4_32_12$, with the following cell dimensions: $a = b = 65.2$ and $c = 169.0$ Å and $a = b = 65.2$ and $c = 169.3$ Å, respectively.

X-ray data collection and refinement. The data sets of the enzyme-Sar-TBODA and enzyme-Ala-TBODA complexes were collected to 2.4 Å resolution at room temperature using wavelengths of 1.00 and 1.15 Å from a synchrotron radiation source with an ADSC Quantum4R CCD detector on beamline 6A and 18B stations at the Photon Factory (Tsukuba, Japan). The crystals were enclosed within glass capillaries with a small amount of mother liquor. In the data collection of enzyme-Sar-TBODA complexes, 80 frames with an oscillation angle of 0.5° were taken by an exposure time of 20 s per frame while in the case of enzyme-Ala-TBODA complexes, 100 frames with an oscillation angle of 1.0° were taken by an exposure time of 5 s per frame. The diffraction data sets were processed and scaled by MOSFLM and SCALA in the CCP4 program suite (5) and HKL2000 programs (20). The crystals showed decay upon exposure. The mean intensity of the last frame was reduced to 50% on enzyme-Sar-TBODA crystal and to 40% on enzyme-Ala-TBODA crystal, estimating from the scale factors. Although the intensity decreased, the crystals showed no significant change in cell parameters and the mosaicities. We used scaling data to 2.4 Å resolution as reliable data for structure determination.

The crystals of inhibitor complexes were isomorphous with those of the wild-type prolyl aminopeptidase. Therefore, the coordinates of 1QTR (24) were used as the first model for refinement. The enzyme-Sar-TBODA complex was refined using rigid-body refinement, simulated annealing, and energy minimization by the Crystallography & NMR System (CNS) program (4) using diffraction data from 30 to 2.4 Å resolution. The model was corrected by inspecting the composite omit map using the program XtalView (18). A difference Fourier map displayed a residual electron density map corresponding to Sar-TBODA at the active site. Water molecules were picked up on the basis of the peak height and the distance criteria from the difference map. Water molecules whose thermal factors were above 60 Å 2 after refinement were removed from the list. Several rounds of refinement cycles and model building resulted in an R_{factor} of 19.1% and an R_{free} of 22.6%. In a similar fashion, the enzyme-Ala-TBODA complex was refined using data from 30 to 2.4 Å resolution. The model, which contained a protein chain, Ala-TBODA, and water molecules, was refined, resulting in an R_{factor} of 17.8% and an R_{free} of 21.7%. A structure diagram was drawn using the MOLSCRIPT (16), Raster3D (19), and POVscript $^+$ (6) programs. The detection and volume of substrate binding pocket was calculated using the VOIDOO (15) program. Superimposition of each protein was carried out by LSQKAB in the CCP4 program suite (5).

Construction of enzyme-Hyp-TBODA and enzyme-AcHyp-TBODA complex models. The docking models complexed with putative inhibitors of Hyp-TBODA and AcHyp-TBODA were calculated by a conjugate gradient minimization method using the CNS program. The initial models were constructed on the basis of enzyme-Pro-TBODA complex (Protein Data Bank code 1WM1) using the

TABLE 1. Data collection and refinement statistics of the inhibitor complexes a

Statistic	Value for:	
	Ala-TBODA	Sar-TBODA
Data collection		
Resolution range (Å)	50–2.4 (2.49–2.40)	30–2.4 (2.53–2.40)
Space group	$P4_32_12$	$P4_32_12$
a, b (Å)	65.2	65.2
c (Å)	169.3	169.0
Wavelength (Å)	1.1500	1.0000
No. of unique reflections	15,059 (1,467)	14,640 (2107)
Completeness (%)	99.8 (100)	98.2 (99.6)
R_{merge} (%)	6.6 (28.3)	5.9 (22.2)
Mean I/σ (I)	45.4 (10.3)	14.1 (4.6)
Redundancy	7.7 (7.9)	2.9 (2.9)
Refinement		
Resolution range (Å)	30–2.40	30–2.40
No. of reflections used in refinement	14,999	14,599
R factor (%)	17.7	19.1
R_{free} (%)	21.7	22.6
RMSD bonds (Å)	0.005	0.006
RMSD angles (°)	1.16	1.20

a Values in parentheses refer to the last resolution shell. RMSD, root mean square deviation. $R_{\text{merge}} = \sum_{\text{hkl}} \sum_i |I_{\text{hkl},i} - \langle I_{\text{hkl}} \rangle| / \sum_{\text{hkl}} \sum_i I_{\text{hkl},i}$, where I = observed intensity and $\langle I \rangle$ = average intensity for multiple measurements.

XtalView program. During the minimization, not all atoms were fixed, but the protein chain was harmonically restrained. The root mean square (RMS) deviations of atomic distance were calculated on residues involved in the binding site between each model and the enzyme-Pro-TBODA complex, resulting in 0.08 and 0.09 Å on enzyme-Hyp-TBODA and enzyme-AcHyp-TBODA complex models, respectively.

Coordinates. The atomic coordinates and structure factors (codes 1X2B and 1X2E) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

RESULTS

X-ray crystallographic analyses of enzyme-Ala-TBODA and enzyme-Sar-TBODA complexes. We determined the structure of the *S. marcescens* prolyl aminopeptidase complexed with Pro-TBODA (1WM1) in our previous paper (8). This enzyme is specific to the N-terminal proline residue. However, it shows a little activity toward Ala- β NA and Sar- β NA. Alanine and sarcosine residues are smaller than proline residues, and their side chains can be considered parts of the pyrrolidine ring. To clarify the details of the molecular recognition mechanism of prolyl aminopeptidase, the present study examined the crystal structures of the enzyme complexed with two inhibitors: Ala-TBODA and Sar-TBODA. The $|F_o| - |F_c|$ omit maps for Ala-TBODA and Sar-TBODA are shown in Fig. 1A and B, respectively. The refined model of enzyme-Ala-TBODA contains a monomer of 313 amino acid residues, Ala-TBODA, and 75 water molecules with an R_{factor} of 17.7% at 2.4 Å resolution (Table 1). This model lacks four residues: three in the N terminus and one in the C terminus. The average thermal factor of main chain atoms is 33 Å 2 . Analysis of the stereochemistry with PROCHECK (17) showed that all the main chain atoms fall within the generously allowed region of the Ramachandran plot, with 244 residues (91.4%) in the most

FIG. 2. Stereograms of the substrate binding pocket at the active site. (A) The hydrophobic pocket was drawn as emerald surface with stick models of Pro-TBODA (orange), Ala-TBODA (green), and Sar-TBODA (sky blue). (B) Computer-designed enzyme-AcHyp-TBODA complex model. It was suggested that the 4-acetyloxy group was fitted into the unusual extra space. The figures were drawn using the POVscript⁺ and Raster3D programs.

avored region, 22 residues (8.2%) in the additionally allowed region, and the Ser113 residue in the generously allowed region. The enzyme-Sar-TBODA complex contains 313 amino acid residues, Sar-TBODA, and 43 water molecules with an R_{factor} of 19.1% at 2.4 Å resolution. The model lacks four residues (in the N and C termini), and the average thermal factor of main chain atoms is 37 Å². Analysis of the stereochemistry with PROCHECK showed that 242 (90.6%) and 24 (9.0%) residues fall within the most favored and the additionally allowed regions of the Ramachandran plot, respectively. The Ser113 residue was in the generously allowed region.

Unusual extra space at the active site. The structures of the enzyme-Ala-TBODA and enzyme-Sar-TBODA complexes were superimposed on that of the enzyme-Pro-TBODA complex using the least-squares method. The C α atoms of the enzyme-Ala-TBODA complex were superimposed on those of the enzyme-Pro-TBODA complex within an RMS deviation of 0.11 Å, and those of the enzyme-Sar-TBODA complex were also superimposed within a 0.09-Å RMS deviation. Although the C α positions differed slightly in the position of Ser213-Glu215 (0.52 to 1.02 Å) among these complexes, the overall structures were identical. As shown in Fig. 1C, the positions of

TABLE 2. Hydrolysis of substrates containing proline and its derivatives by prolyl aminopeptidases from *S. marcescens* and *B. coagulans*^a

Substrate	S1 and S1' sites	Substrate specificity value for:	
		<i>S. marcescens</i> ($\mu\text{mol/min/mg}$)	<i>B. coagulans</i> ($\mu\text{mol/min/mg}$)
Pro- β NA		0.80 (100)	54.5 (100)
Hyp- β NA		0.21 (26)	0.63 (1.2)
AcHyp- β NA		8.34 (1,037)	0.25 (0.46)

^a Numbers in parentheses show relative values.

the N-terminal Ala and Sar residues each fit in the position of the Pro residue, although the positions of the inhibitor oxadiazole moiety in the other two complexes were different from that in the enzyme-Pro-TBODA complex. In the enzyme-Pro-TBODA complex, the distances between the N-terminal atom of the inhibitor to Glu204 (OE1 and OE2) and Glu204 (OE1) were 3.12, 3.23, and 2.85 Å, respectively. In the enzyme-Ala-TBODA and enzyme-Sar-TBODA complexes, these distances were 2.46, 2.96, and 2.72 Å (enzyme-Ala-TBODA), and 3.11, 3.43, and 3.15 Å (enzyme-Sar-TBODA), respectively. The distances in the enzyme-Ala-TBODA complex were shorter than those in the other complexes. It was considered that the ion pair bonding between the amino group of Ala and each glutamate residue was formed without steric hindrance since the N terminus of Ala-TBODA is a free amino group, unlike the other N termini. Figure 2A shows the three superimposed complexes at the cavity using a surface model. There was an unusual extra space with about 50 Å³ at position 4 of the pyrrolidine ring of the proline residue.

Substrate specificity for proline, hydroxyproline, and acetyloxyproline. The substrate specificity of prolyl aminopeptidase from *S. marcescens* was compared with that of the enzyme from a nonpathogenic strain of *B. coagulans* using Pro- β NA, Hyp- β NA, and AcHyp- β NA (Table 2). Hyp- β NA was a poorer substrate than Pro- β NA for both enzymes. The acetylated form of Hyp- β NA was, however, the best substrate for the enzyme from *S. marcescens*, while Pro- β NA was the best for the enzyme from *B. coagulans* among the substrates tested. These results show that hydroxyproline is resistant to hydrolysis by these enzymes and that the acetyl derivative of hydroxyproline is the favored substrate for the enzyme from *S. marcescens*.

To confirm the data from the kinetic study of 4-hydroxyproline and 4-acetyloxyproline for the enzyme from *S. marcescens*, docking models were constructed to adopt conjugate gradient minimization using the structure of the enzyme-Pro-TBODA

complex as the base model. As shown in Fig. 2B, both the hydroxyl group and the 4-acetyloxy group fit into this space. The models of the enzyme-Hyp-TBODA and enzyme-AcHyp-TBODA complexes were superimposed on the enzyme-Pro-TBODA complex. Although they are almost identical to the enzyme-Pro-TBODA complex, these models had a slight difference in conformation of residues at the substrate binding site. In comparison to the enzyme-Pro-TBODA complex, the side chain of Phe139 in the enzyme-AcHyp-TBODA model, which was involved in the hydrophobic pocket, shifted in a direction away from the inhibitor; the distance between C ϵ atoms was 0.35 Å. In the enzyme-Hyp-TBODA model, however, the side chain position of Phe139 was not significantly changed. The position of AcHyp moiety was well fit into that of Pro moiety, while Hyp moiety changed the position by a turn of about 20° on the C α atom. Accordingly, the hydroxyl group of Hyp was located from the carbonyl group of Ala270 to the distance of 2.85 Å, which is within the hydrogen bonding range.

Analysis of the role of each residue by site-directed mutagenesis. In addition to the interaction bonds, the crystal structure of the complex revealed a hydrophobic pocket consisting of Phe139, Tyr149, Tyr150, Phe236, and Cys271 (Fig. 3). Each of these was changed to alanine to produce the F139A, Y149A, Y150A, F236A, and C271A mutants, respectively, by site-directed mutagenesis. Each mutant was purified by the standard method. Table 3 summarizes the kinetic constants, including K_m , k_{cat} , and the catalytic efficiency (k_{cat}/K_m) of each mutant obtained using Pro- β NA, Hyp- β NA, and AcHyp- β NA as substrates. Each mutation resulted in a large decrease in k_{cat}/K_m except for that obtained for the C271A mutant with Hyp- β NA as a substrate. With respect to Pro- β NA and AcHyp- β NA, the best substrates for the two enzymes, the k_{cat}/K_m values obtained for the F139A, Y149A, and F236A mutants were more than 10-fold lower than those for the wild type. The k_{cat}/K_m values of the F139A and F236A mutants were lower for Hyp- β NA than those for AcHyp- β NA, while the k_{cat}/K_m values of the Y149A and C271A mutants were higher for Hyp- β NA than those for AcHyp- β NA.

DISCUSSION

By our X-ray crystallographic study of the enzyme and Pro-TBODA complex, the pyrrolidine ring of the proline residue was fitted into the hydrophobic pocket, and the amino group of the proline turned out to be fixed with two glutamic acids by a salt bridge. Site-directed mutagenesis confirmed the contributions of Phe139, Phe236, and Tyr149 to the hydrophobic pocket, as well as those of Glu204 and Glu232 to the salt bridge formation (8, 9). The present study focuses on the interface between the enzyme and the substrate.

Most proline-specific peptidases showed alanyl activity, although the rates of hydrolysis varied. Dipeptidyl aminopeptidase IV (23), prolyl oligopeptidase (23), and prolyl aminopeptidase from *B. coagulans* (13) exhibited hydrolytic activity of the Ala-X bond at rates 10 times lower than those of the Pro-X bond. Interestingly, the rate of hydrolysis of the Ala-X bond by prolyl aminopeptidase from *S. marcescens* was half that of the Pro-X bond. Dipeptidyl aminopeptidase II acts on the Pro-X and Ala-X bonds with almost equal rates of hydrolysis (7).

FIG. 3. Stereogram of the enzyme–AcHyp-TBODA complex model, which was drawn using the MOLSCRIPT and Raster3D programs, viewed from the bottom of the hydrophobic pocket. 4-Acetyloxyproline is shown in pink, and the residues around the active site are shown as stick models.

Moreover, prolyl aminopeptidase recognizes the pyrrolidine ring conformation. The enzyme can accept Sar, L- α -aminobutyryl, and norvalyl- β NA as substrates because they could partially mimic the pyrrolidine ring. The same results were obtained by the inhibitors Pro-TBODA, Ala-TBODA, and Sar-TBODA (8). These results indicate that the enzyme has a space for a five-membered ring group at the S1 site of the enzyme and that the substrate is recognized in this cavity. To confirm this hypothesis, the enzyme–Ala-TBODA and enzyme–Sar-TBODA complexes were analyzed by X-ray crystallography. The $|F_o| - |F_c|$ electron density maps of these complexes are shown in Fig. 1, and the alanine and sarcosine residues of inhibitors were superimposed into the pyrrolidine ring of the proline residue. This result clearly shows that the hydrophobic space fits with a pyrrolidine ring and that the salt bridges recognize the α -amino group. Proline and two other amino acids (alanine and sarcosine), all sharing a part of the pyrrolidine ring, are recognized in essentially the same manner. This recognition seems to explain why sarcosine oxidase acts toward proline. This is the first study to recognize sarcosine and alanine residues as parts of a proline residue by X-ray crystallography.

Kinetic analyses, together with computer docking studies,

sometimes provide interesting information about fine structures that are rather hard to notice by general observation. Such an example is described below. Hyp- β NA and AcHyp- β NA were synthesized as substrates. The k_{cat}/K_m value of Hyp- β NA was 20-fold lower than that of Pro- β NA, while that of AcHyp- β NA was 4-fold higher than that of Pro- β NA. It is evident that the more hydrophobic AcHyp- β NA is a favored substrate. Interestingly, there is an unusual extra space at the bottom of the hydrophobic pocket where the proline residue is fixed in the prolyl aminopeptidase (Fig. 2A). Based on the structural data on the enzyme–Pro-TBODA complex by X-ray crystallography (8), we constructed the most reliable conformations of putative inhibitor Hyp-TBODA and AcHyp-TBODA docking at the active site using the XtalView and CNS programs. As predicted, the 4-acetyloxy group of AcHyp-TBODA fit into the unusual extra space behind the hydrophobic pocket (Fig. 2B).

When Hyp- β NA was docked into the active site, the pyrrolidine ring position became different from that of Pro- β NA, probably due to the hydrophobic effect of Tyr150 against the hydroxyl group of the substrate. The change in the substrate conformation can explain the large decrease in K_m for Hyp- β NA as well as the low catalytic efficiency (k_{cat}/K_m) because

TABLE 3. Kinetic constants for the wild type and mutants of prolyl aminopeptidase

Enzyme	Pro- β NA			Hyp- β NA			AcHyp- β NA		
	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)
Wild type	0.51	9.70	19.0	9.3	3.9	0.42	1.64	84.1	51.2
F139A mutant	1.17	0.69	0.59	0.83	0.063	0.076	1.45	5.85	4.02
F236A mutant	1.31	0.35	0.27	0.84	0.017	0.020	1.60	3.52	2.20
Y149A mutant	0.42	0.63	1.48	1.13	0.12	0.11	2.92	0.88	0.33
Y150A mutant	LA ^a	LA	LA	LA	LA	LA	LA	LA	LA
C271A mutant	1.58	7.09	4.50	4.27	1.79	0.42	2.67	4.93	1.84

^a LA, low activity.

the distance between the carbonyl carbon and the hydroxyl group of the active site Ser113 increases. Most notably, the k_{cat} value for AcHyp- β NA is almost 10 times that for Pro- β NA. We do not have a plausible explanation for this high value. As we reported previously, the prolyl aminopeptidase showed twice the k_{cat} value toward Ala- β NA than toward Pro- β NA, although the k_{cat}/K_m value for Ala- β NA was less than half of that for Pro- β NA because of the high K_m value for Ala- β NA. A comparison of the Pro-TBODA and the Ala-TBODA structures clarified in this study reveals that the positions of the carbonyl group of the scissile peptide bond are significantly different from each other. The positioning of the substrate could be affected by the presence of an acetyloxy group to increase catalytic activity. To clarify the exact mechanism underlying the high k_{cat} value toward AcHyp- β NA, detailed X-ray crystallographic studies on the inactive enzyme complexed with a substrate containing the 4-acetyloxy group will be necessary.

Three-dimensional structures of enzyme complexed with proline-containing inhibitor have been clarified in prolyl oligopeptidase (Protein Data Bank code 1U00) and dipeptidyl aminopeptidase IV (1NU8). These proline-specific peptidases were thought to each have a space for a hydroxyproline residue but not for a 4-acetyloxyproline residue. To confirm this, we measured the activity of dipeptidyl aminopeptidase IV from pig kidney for Gly-Hyp- β NA and for Gly-Pro- β NA. The enzyme hydrolyzed the former at a rate 50 times lower than that of the latter, as prolyl aminopeptidase does. However, the enzyme showed absolutely no activity toward Gly-AcHyp- β NA (unpublished data).

Since a clear difference in substrate specificity for AcHyp- β NA was observed between the enzymes from *S. marcescens* and *B. coagulans* (14) (Table 2), a homology search was carried out using DDBJ BLAST. Many enzyme genes appeared homologous to the enzyme from *S. marcescens*. They include hypothetical genes in the genomes of many pathogenic bacteria. However, fewer enzyme genes had a significant homology to the enzyme from *B. coagulans*.

To elucidate the role of amino acid residues forming the hydrophobic pocket, kinetic parameters were determined for the F139A, Y149A, F236A, and C271A mutants using Pro- β NA, Hyp- β NA, and AcHyp- β NA as substrates (Table 3). All of these residues are well conserved among the homologous enzyme sequences but are not conserved at all in the *B. coagulans*-type prolyl aminopeptidases. The Y150A mutant showed a very low level of activity. Since Tyr150 is surrounded by Phe236, Tyr149, Trp200, and Trp203 (Fig. 3) and exists as a central core of the bottom wall of the hydrophobic pocket, replacing Tyr with Ala may have resulted in a loss of stability, possibly through a conformational change at the hydrophobic pocket.

We have already reported the importance of Phe139, Tyr149, and Phe236 for proline recognition (8). In this study, we also demonstrated the importance of Tyr150 and Cys271. K_m values for Hyp- β NA were decreased by all the mutations, while those for Pro- β NA and AcHyp- β NA were increased or not changed very much, indicating that these residues are important for a hydrophobic environment for proline. If the space is responsible only for the activity against AcHyp- β NA, the activity ratios against Pro- β NA and AcHyp- β NA should

not change very much because alanine-substituted mutants can provide more space for substrates. The k_{cat}/K_m values of the F139A and F236A mutants were lower for Hyp- β NA than those for AcHyp- β NA, while the k_{cat}/K_m value ratios of the Y149A and C271A mutants were reversed; Pro- β NA was preferred over AcHyp- β NA. This may suggest that the Tyr149 and Cys271 residues have special roles in binding or orienting an acetyl derivative of the hydroxyproline-containing substrates. Most of the residues forming the hydrophobic pocket are located on the helix domain (α_{1-6}) of the enzyme and are well conserved among homologous enzymes, as mentioned above. These homologous enzymes are likely to have AcHyp- β NA-hydrolyzing activity as the *S. marcescens* enzyme does.

It was difficult to align the amino acid sequences of prolyl aminopeptidases with that of the *B. coagulans* enzyme. However, since the catalytic domain showed significant homology, the *B. coagulans* enzyme must have a similar catalytic domain with a different helix domain, giving strict proline specificity over hydroxyproline or other derivatives.

Several types of acetyltransferases in organisms have been found and studied. Although there is no report on hydroxyproline acetyltransferase in *Serratia*, there may be a possibility that an acetylation of hydroxyproline is a necessary step for an efficient degradation of collagen by some pathogenic bacteria.

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