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Substrate Recognition Mechanism of Prolyl Aminopeptidase from *Serratia marcescens*[1]

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Molecular cloning of the gene and the crystal structure of the prolyl aminopeptidase [EC 3.4.11.5] from *Serratia marcescens* have been studied by us [*J. Biochem.* 122, 601–605 (1997); *ibid.* 126, 559–565 (1999)]. Through these studies, Phe139, Tyr149, Glu204, and Arg136 were estimated to be concerned with substrate recognition. To elucidate the details of the mechanism for the substrate specificity, the site-directed mutagenesis method was applied. The F139A mutant showed an 80-fold decrease in catalytic efficiency (k_{cat}/K_m), but the Y149A mutant did not show a significant change in catalytic efficiency. The catalytic efficiency of the E204Q mutant was about 4% of that of the wild type. The peptidase activity of the mutant (R136A) was markedly decreased, however, arylamidase activity with Pyr-βNA was retained as in the wild-enzyme. From these results, it was clarified that the pyrrolidine ring and the amino group of proline at the S1 site were recognized by Phe139 and Glu204, respectively. P1′ of a substrate was recognized by Arg136. On the other hand, the enzyme had two cysteine residues. Mutants C74A and C271A were inhibited by PCMB, but the double mutated enzyme (C74/271A) was resistant to it.

Key words: aminopeptidase, peptidase, proline, prolyl aminopeptidase, *Serratia marcescens*, substrate recognition.

Prolyl aminopeptidase (pap, proline iminopeptidase, EC 3.4.11.5) catalyzes the removal of the N-terminal proline from peptides. This enzyme, from *Escherichia coli*, was studied first by Sarid *et al.* (*1*), and has been purified from bacteria and plants, however, its existence in animals is not certain (*2*). Several *pap* genes, from *Aeromonas sobria* (*3*), *Bacillus coagulans* (*4*), *Flavobacterium meningosepticum* (*5*), *Hafnia alvei* (*6*), *Lactobacillus delbrueckii* subsp. *Bulgaricus* (*7*), *Neisseria gonorrhoeae* (*8*), *Thermoplasma acidophilum* (*9*), and *Xanthomonas campestris* pv. *citri*, have been cloned and sequenced (*10*). Since the prolyl aminopeptidase is inhibited by *p*-chloromercuribenzoic acid (PCMB) but not by diisopropyl fluorophosphate (DFP), the enzyme was reported to be a sulfhydryl enzyme (*11*, *12*). However, the presence of a typical consensus sequence, GXSXG, around the serine residue of the catalytic triad was observed on site-directed mutagenesis, and then prolyl aminopeptidase was classified as a serine enzyme (*13*). Recently, the enzyme gene from *Serratia marcescens*, *spap*, was cloned (*14*), and its three-dimensional structure was solved by X-ray crystallography with the multiple isomorphous replacement method by our group (*15*).

In this study, the mechanism of the substrate specificity for proline residues and the effect of a heavy metal on the enzyme were studied by means of site-directed mutagenesis.

MATERIALS AND METHODS

Materials—Restriction endonucleases and other modifying enzymes were purchased from Toyobo Biochemicals. or New England Biolabs. Proline β-naphthylamide (Pro-βNA), L-prolyl-L-alanine (Pro-Ala), and Fast Garnet GBC were from Sigma Chemical. The oligonucleotide primers for site-directed mutagenesis were synthesized by Amersham Pharmacia Biotech. The Mutan-Super Express Km kit was from TaKaRa Shuzo. The ALFexpress AutoCycle sequencing kit and other reagents for DNA sequencing were obtained from Amersham Pharmacia Biotech.

Bacterial Strains, Plasmids, and Media—*E. coli* MV1184 (*ara*, Δ(*lac-proAB*), *rspL*, *thi*(*ϕ80d lacZ*ΔM15), Δ(*srl-recA*) 306::Tn10 (*tet*^{r})/F′[*traD*36, *proAB*+, *lacI*^{q}, *lacZ*ΔM15]) was used for site-directed mutagenesis. *E. coli* DH1 (*supE*44, *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1), and DH5α [*supE*44, Δ*lac*U169(*ϕ80d lacZ*ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1] were used as hosts for expression. Plasmids pUC19 and pKF19k were used as vectors. Bacteria were grown in Luria-Bertani broth [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 antibiotic concentrations used in this

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Abbreviations: DTNB, 5′-dithio-bis-2-nitrobenzoic acid; EDTA, ethylenediaminetetraacetic acid; LA-PCR, long and accurate polymerase chain reaction; dNTP, deoxynucleoside triphosphate; ODA, oligonucleotide-directed dual amber; Pro-βNA, L-prolyl-β-naphthylamide; PCMB, *p*-chloromercuribenzoic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

study were as follows: ampicillin, 100 μg/ml (LB-broth) or 50 μg/ml (N-broth); kanamycin, 50 μg/ml.

Construction of Mutant Enzymes by Site-directed Mutagenesis—A Mutan-Super Express Km kit based on the ODA (Oligonucleotide-directed Dual Amber)-LA PCR method (*15, 16*) was used for the construction of seven mutants; R136A, F139A, Y149A, E204Q, C74A, C271A and C74/271A. PCR was carried out in a 50 μl reaction mixture comprising 5 pmol selection primer, 5 pmol mutation primer, 10 ng of template DNA, 5 μl of 10 LA PCR Buffer (Mg^{2+} plus), 8 μl of a deoxynucleoside triphosphate (dNTP) mixture, 2.5 mM each, and 2.5 units of TaKaRa LA Taq DNA polymerase. The amplification conditions comprised up to 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 3 min. The mutation primers are shown in Table I. After the PCR cycles, the reaction mixture was allowed to cool at 4°C.

The DNA in the reaction mixture was precipitated by adding 5 μl of 3 M sodium acetate and 125 μl of 100% cold ethanol. The precipitate was washed twice with 70% cold ethanol, dried and then dissolved in 5 μl of sterilized water. This DNA solution containing plasmid DNA was used to transform *E. coli* MV1184, and the transformants were selected on LB plates containing kanamycin. Each of the mutations was confirmed by sequence analysis, using an ALFexpress DNA Sequencer. DNA fragments containing individual mutations were prepared by digesting the respective plasmids with *Eco*RI and *Pst*I (for pSPAP-M1), or *Eco*RI and *Eco*RV (for pSPAP-M2). These fragments were inserted into the same site of pUC19 (for pSPAP-M1), or pSPAP-HE (for pSPAP-M2), and then the cloned plasmids were used to transform *E. coli* DH1 or DH5α for expression.

Purification of the Mutant Enzymes—*E. coli* DH1 or DH5α cells harboring individual mutant plasmids were aerobically cultured in 12 liters of N-broth containing ampicillin at 37°C for 17 h, in a jar fermentor. Cells were collected by centrifugation and disrupted with a Dyno Mill, and then cell debris was removed by centrifugation. The clear supernatant was treated with protamine sulfate (15 mg/g of wet cells), and the resulting mixture was kept for 15 min and then centrifuged at 9,000 rpm for 20 min. The supernatant was fractionated with ammonium sulfate at 25 to 55% saturation. The precipitate was dissolved in 20 mM Tris-HCl buffer (pH 8.0). The resulting solution was desalted, applied to a DEAE-Toyopearl column and then eluted with a linear gradient of 0 to 0.5 M sodium chloride. The active fractions were combined and concentrated by ultrafiltration, using an Amicon 8200, Grace. The concentrated solution was 25% saturated with ammonium sulfate, and then applied to a column of Toyopearl HW65C equilibrated with 20 mM Tris-HCl buffer (pH 8.0) 25% saturated with ammonium sulfate. The adsorbed enzyme was eluted with a decreasing linear gradient of ammonium sulfate concentration from, 25 to 0% saturation, in 20 mM Tris-HCl buffer (pH 8.0). The enzyme in the active fractions was concentrated by ultrafiltration, using an Amicon 8200, and then dialyzed against 20 mM Tris-HCl buffer (pH 8.0) and Milli-Q water.

TABLE I. **Primers used for site-directed mutagenesis.**

Mutant	Primer
Arg136 Ala (R136A)	ATGGTGCTGGCCGGCATCTTC
Phe139 Ala (F139A)	CGCGGCATCGCCACCTGCGC
Tyr149 Ala (Y149A)	CTGCATTGGGCTTACCAGGAC
Glu204 Gln (E204Q)	AGCGTGTGGCAAGGGGAGACG
Cys74 Ala (C74A)	CAGCGCGGTGCCGGCCGCTCC
Cys271 Ala (C271A)	GACATGGCCGCCCAGGTGCAG

Assaying of Prolyl Aminopeptidase Activity and Kinetic Studies—The prolyl aminopeptidase activity was assayed using Pro-βNA and Pro-Ala as substrates. The former substrate was used for the general assay method (*12*).

(1) Pro-βNA substrate: 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 2 mM Pro-βNA. After 5 min (unless otherwise indicated) incubation at 37°C, the reaction was stopped by adding 1 ml of a 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 20 min, using a spectrophotometer. One unit of the activity was defined as the amount of the enzyme which released 1 μmol of β-NA per min under the above conditions.

(2) Pro-Ala substrate: The reaction mixture consisted of 0.1 ml of enzyme solution and 0.1 ml of 3 mM Pro-Ala. After 3 min incubation at 37°C, the reaction was stopped by adding 0.8 ml of 1 M sodium acetate buffer (pH 2.8). To the mixture was added 0.1 ml of 10% ninhydrin in 95% ethanol followed by incubation at 70°C for 10 min. Then, the absorbance at 440 nm was measured.

The protein concentration was determined by the method of Bradford and from the absorbance at 280 nm ($E_{1\%}$ = 10.0). To determine the K_m values, the substrate concentration was varied. Lineweaver-Burk plots were used to calculate K_m and apparent V_{max}. To calculate k_{cat}, the subunit molecular weight of wild type prolyl aminopeptidase, 36,083, was used.

Analysis of Inhibition by a Reagent Containing Hg—In order to check the inhibitory effect on the mutant of a reagent containing Hg, the wild type and mutant enzyme with the Hg-contained reagent was assayed. The reaction mixture contained 0.8 ml of 100 mM Tris-HCl (pH 8.0) containing PCMB (final, 1–20 μM), 0.1 ml of enzyme solution, and 0.1 ml of 2 mM Pro-βNA. The assay method was the same as described previously.

Quantitative Analysis of Cysteine Residues—In order to confirm the number of cysteine residues in the mutant enzyme, analysis of free SH groups was carried out with an Ellman reagent (DTNB) (*18*). The reaction mixture comprised 2.5 ml of 100 mM Tris-HCl (pH 8.0) containing 0.02 μmol enzyme and 8 M urea, and 0.1 ml of a 0.01 M DTNB solution in K-phosphate buffer (pH 7.0). The OD_{412nm} reflecting the amount of quantitative reaction product was read from the reaction mixture, using a spectrophotometer.

RESULTS

S1 Site of Prolyl Aminopeptidase—Since prolylaminopeptidase specifically removes the amino terminal proline residue from substrates, the pyrrolidine ring and the imido group must be recognized by the enzyme. The three-dimensional structure of the active site of prolyl aminopeptidase is shown in Fig. 1. From the structure, we estimated that the pyrrolidine ring was recognized by Phe139 and Tyr149, and the imido grop by Glu204. Then, in order to confirm our estimation, Phe139 and Tyr149 were changed to Ala to

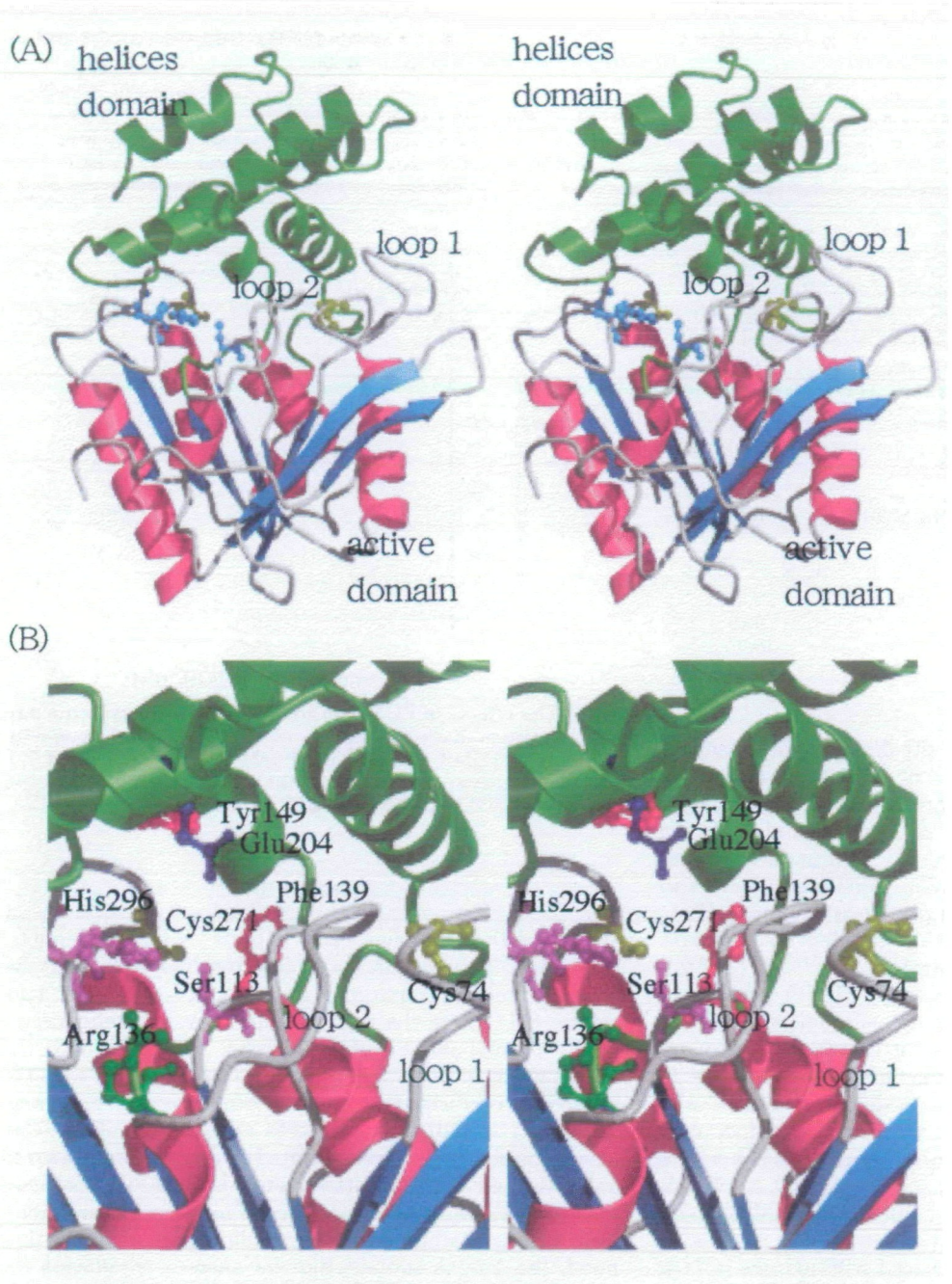

Fig. 1. Stereo view of the whole (a) and active site (b) structures of prolyl aminopeptidase. The catalytic triad and target residues for site-directed mutagenesis are shown as a ball-and-stick model. This figure was produced with the program MOLSCRIPT (*19*), and rendered with Raster3D (*20*).

yield F139A and Y149A, and Glu204 to Gln to produce E204Q. Mutated *pap* genes inserted into pUC19 were overexpressed in *E. coli* cells. All of the mutant enzymes were purified from cell-free extracts of *E. coli* DH1 or DH5α cells by ion-exchange and hydrophobic interaction chromatographies on DEAE-Toyopearl and Toyopearl HW65C, respectively. As shown in Fig. 2, the enzymes were purified homogeneously.

The kinetic parameters of each mutant, including K_m, k_{cat}, and catalytic efficiency (k_{cat}/K_m) as to Pro-βNA, were determined (Table II). Among alanine-substituted enzymes, the k_{cat}/K_m value of F139A decreased by 80-fold. This was due to changes in both K_m and k_{cat}. Phe139 seems to be a major contributor to the hydrophobic pocket. Tyr149 also seems to be a component of the hydrophobic pocket. The Y149A mutant showed little decrease in the k_{cat}/K_m value. However, the K_m and k_{cat} values were significantly changed, suggesting a certain involvement of Tyr149 in substrate binding. For the E204Q mutant, a dramatic decrease in k_{cat} was observed and the k_{cat}/K_m value was only 4% of that of the wild type enzyme. The K_m value increased by 2-fold. Since Glu204 is expected to interact with the imido group of the substrate proline residue, it was indicated that the mutation of this residue to glutamine affected the interaction between the substrate and the enzyme.

S1′ Site of Prolyl Aminopeptidase—As shown in Fig. 1, Arg136 was found near catalytic Ser113 in the three-dimensional structure. With the catalytic triad at the center, this position is on the opposite side of the hydrophobic pocket into which the terminal proline residue can fit. It is possible that Arg136 may interact with P1′ of a substrate. In order to clarify the role of Arg136 it was replaced with

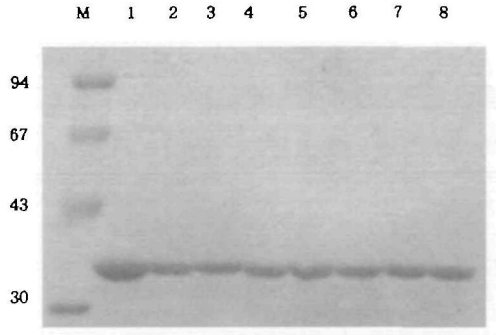

Fig. 2. **SDS-PAGE of the purified mutant prolyl aminopeptidases.** Lane M, marker proteins; lane 1, wild type; lane 2, R136A; lane 3, F139A; lane 4, Y149A; lane 5, E204Q; lane 6, C74A; lane 7, C271A; lane 8, C74/271A.

TABLE II. **Kinetic constants for the wild type prolyl aminopeptidase and its mutants.**

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Wild type	0.42	9.81	23.4
F139A	1.38	0.41	0.29
Y149A	0.07	1.40	19.9
E204Q	0.82	0.08	0.097

alanine (R136A) by means of site-directed mutagenesis. The mutant enzyme was purified by the standard method. The purity of the enzyme was checked by SDS-PAGE (Fig. 2). Kinetic parameters of the R136A mutant and wild type enzymes were determined. Table III summarizes the results. Both enzymes showed similar kinetic parameters to arylamidase when Pro-βNA was used as a substrate. However, the mutant enzyme (R136A) exhibited no peptidase activity with Pro-Ala as the substrate.

The Cysteine Residues Involved in the Sensitivity to PCMB—In the earlier study, prolyl aminopeptidase was classified as a cysteine enzyme, as it was very sensitive to PCMB. The purification was carried out in the presence of 2-mercaptoethanol. There are two cysteine residues, Cys74 and Cys271, in the wild type enzyme (Fig. 1). Since it is now established that the prolyl aminopeptidase is a serine enzyme and is little inhibited by iodoacetic acid, inhibition by PCMB is a secondary effect. In order to determine which cysteine residue(s) is involved in the inhibition by PCMB, both Cys74 and Cys271 were replaced with alanine (C74A, C271A, and C74A/271A) by means of site-directed mutagenesis. The mutant enzymes were purified by the same method. The purity of the enzymes was checked by SDS-PAGE (Fig. 2). The cysteine content of each mutant was measured with Ellman reagent. The C74A, C271A, and C74/271A mutants had 1.0 mol, 0.9 mol and no cysteine residue per mol of enzyme, respectively.

The kinetic parameters of these three mutant enzymes were almost the same (data not shown). As shown in Fig. 3, both the C74A and C271A mutants were inhibited by PCMB in a similar manner to the wild type enzyme. But the double mutant, C74A/271A, was absolutely resistant to PCMB. These findings show that both cysteine residues were modified by PCMB, thereby affecting the enzyme activity.

TABLE III. **Kinetic constants for the wild type prolyl aminopeptidase and a mutant (R136).**

Enzyme	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Wild type	Pro-βNA	0.42	9.81	23.4
	Pro-Ala	2.07	16.8	8.12
R136A	Pro-βNA	0.46	8.87	19.3
	Pro-Ala		Very low activity	

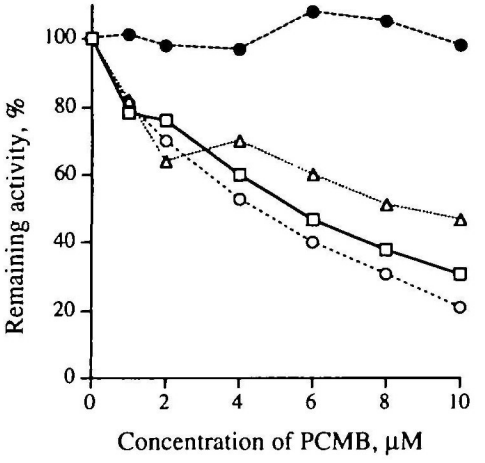

Fig. 3. **The effects of PCMB on the wild type and cysteine mutant enzymes.** Each enzyme was incubated with various concentrateions of PCMB dissolved in 20 mM Tris-HCl buffer, pH 7.0. Remaining activity was assayed by the standard method. Wild type: □, C74A: △, C271A: ○, C74/271A: ●.

DISCUSSION

We found that proline-specific peptidases have a common mechanism for recognizing proline residues in a study involving proline-, sarcosine- and alanine-containing substrates (*21*). From the results of an X-ray crystallographic study of prolyl aminopeptidase from *S. marcescens* (*15*), the hydrophobic pocket was estimated to be concerned with the substrate specificity for the pyrrolidine ring of proline residues. To elucidate the substrate recognition mechanism, Phe139 and Tyr149 were mutated to alanine. As shown in Table II, the F139A mutation resulted in a 80-fold decrease in the k_{cat}/K_m value, indicating that Phe139 plays an important role in the binding of pyrrolidine ring. On the other hand, the Y149A mutant did not show a significant decrease in the k_{cat}/K_m value. However, both K_m and k_{cat} changed. Tyr149 may be involved in orientation of the substrate for cleavage.

Recently, the three-dimensional structure of prolyl oligopeptidase (prolyl endopeptidase EC 3.4.21.26) and the inhibitor (Z-Pro-prolinal) complex was resolved (*22*). In these structures, stacking between the pyrrolidine ring of the proline residue of the inhibitor and the indole ring of Trp595 was indicated to be important for proline specificity. Interestingly, as shown in Fig. 4A, the structure of the α-helix and β-sheet in the active domain of the prolyl oligopeptidase is very similar to that of prolyl aminopeptidase, although little homology was found between the amino acid sequences of these two enzymes. More strikingly, the amino acids of the catalytic triad were located at the same positions in the two enzymes, as shown in Fig. 4B. In the figure, only the proline residue of the inhibitor complexed

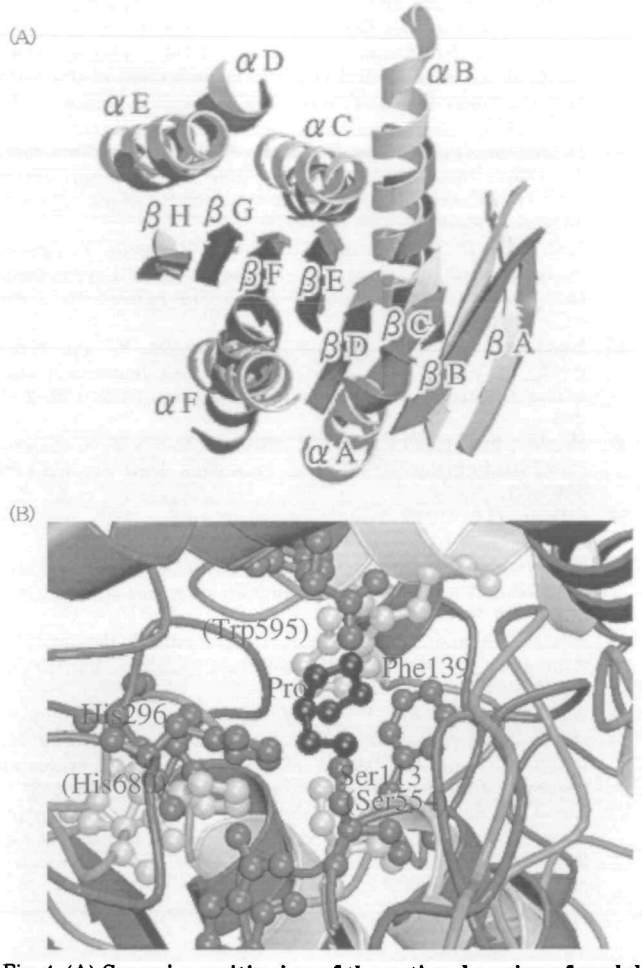

Fig. 4. **(A) Superimpositioning of the active domains of prolyl aminopeptidase (gray) and prolyl oligopeptidase (white). (B) Superimpositioning of the catalytic sites of prolyl aminopeptidase (gray) and prolyl oligopeptidase (white).** Trp595 in prolyl oligopeptidase is stacked with a substrate or inhibitor proline residue (black). The numbers in parentheses are for prolyl oligopeptidase.

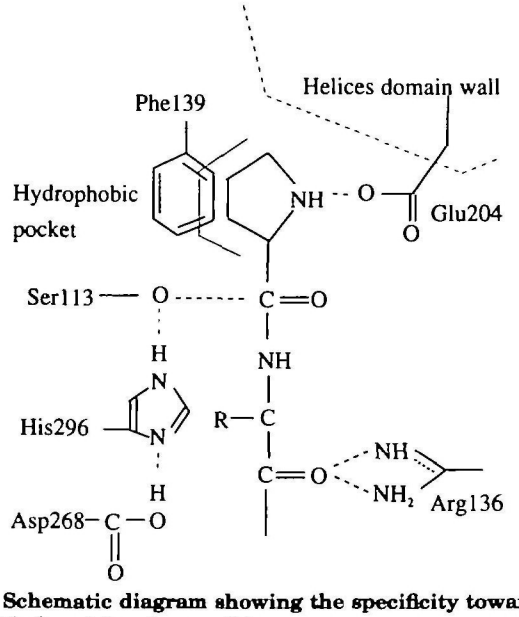

Fig. 5. **Schematic diagram showing the specificity toward P1 and P1′ of prolyl aminopeptidase.**

with prolyl oligopeptidase is superimposed. Almost the same catalytic triad was formed in the two enzymes, although Phe139 in the prolyl aminopeptidase is located on the opposite side of Trp595 of the prolyl oligopeptidase, based on the proline residue of the inhibitor. This observation immediately suggests that the pyrrolidine ring of proline was recognized by stacking with Phe139 and supports the result of kinetic analysis of the F139A mutant of prolyl aminopeptidase.

In addition to the pyrrolidine ring, the imido group must be recognized by the enzyme. For this reason we focused on a glutamate (Glu204) located in the helices domain based on the stereo structure. This glutamate seemed to be important for the binding and neutralization of the charged amino-terminal group of the P1 proline residue. In this study, mutant E204Q was prepared. As a result, the K_m value of the E204Q mutant showed a 2-fold increase, and k_{cat} was decreased and k_{cat}/K_m was about 4% of the wild type value (Table I). This suggests that Glu204 is important for the recognition of the amino terminal proline residue. Since Glu204 is located in the helices domain covering the active center cavity, this residue may have another role for prolyl aminopeptidase to be an exopeptidase.

Arg136 is located at the putative S1′ position and could bind to the carbonyl oxygen of the P1′ amino acid. Interestingly, the mutation of Arg136 to alanine decreased the peptidase activity, but the amidase activity was unaffected. This is due to that Pro-βNA has no carbonyl oxygen at P1′. Thus we confirmed that Arg136 plays a very important role in the recognition of the carbonyl oxygen of the P1′ residue for peptidase activity. These results are summarized in Fig. 5.

As was seen in the F_{EMTS}–F_{native} electron density map with EMTS (ethyl mercury thiosalicylate) (*15*), modification of Cys271 with a heavy metal compound covered the S1 site. However, Cys74 is far from the catalytic triad and S1 site (Fig. 1). The enzyme has a cleft in the catalytic domain at the border with the helices domain. The cleft is in contact with five loops connecting the β-strand and α-helix. The amino acid sequences of these loops are highly conserved in the prolyl aminopeptidases from several origins. Ser113, His296, and Asp268 are located in loop 5, loop 4, and loop 3, respectively. Loop 1 and loop 2 seem to play an important role in making a space for the cavity (Fig. 1). When Cys74, which is located in loop 1, was modified by PCMB, a conformational change of loop 2 was introduced due to the bulky molecule and finally the steric hindrance of the hydrophobic pocket and/or catalytic triad seemed to be produced.

Since similar types of crystals of the mutants to those of the wild-type enzyme were obtained, the basic conformation of each mutant was maintained. To clarify the details of the substrate recognition mechanism, X-ray crystallographic analyses of these mutants are underway.

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