Aminopeptidase N (Proteobacteria Alanyl Aminopeptidase) from *Escherichia Coli*: CRYSTAL STRUCTURE AND CONFORMATIONAL CHANGE OF THE METHIONINE 260 RESIDUE INVOLVED IN SUBSTRATE RECOGNITION*  
Kiyoshi Ito†, Yoshitaka Nakajima†, Yuko Onohara, Masahide Takeo, Kanako Nakashima, Futoshi Matsubara‡, Takashi Ito, and Tadashi Yoshimoto  
Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan  

Aminopeptidase N from *Escherichia coli* is a broad-specific zinc exopeptidase belonging to the aminopeptidase clan MA, family M1. The structure of ligand-free form and enzyme-bestatin complex were determined at 1.5 and 1.6 Å resolution, respectively. The enzyme is composed of four domains: an N-terminal β-domain (Met1-Asp193), a catalytic domain (Phe194-Gly444), a middle β-domain (Thr445-Trp546), and a C-terminal α-domain (Ser547-Ala870). The structure of the catalytic domain exhibits similarity to thermolysin, and a metal binding motif (HEXXHX18E) is found in the domain. The zinc ion is coordinated by His297, His301, Glu320, and a water molecule. The groove on the catalytic domain that contains the active site is covered by the C-terminal α-domain, and a large cavity is formed inside the protein. However, there existed a small hole at the center of the C-terminal α-domain. The amino terminus of bestatin was recognized by residues Glu121 and Glu264, which are located in the N-terminal and catalytic domains, respectively. Glu298 and Tyr381, located near the zinc ion, are considered to be involved in peptide cleavage. A difference revealed between the ligand-free form and the enzyme-bestatin complex indicated that Met260 functions as a cushion to accept substrates with different amino terminal sizes, resulting in the broad substrate specificity of this enzyme.

INTRODUCTION

Aminopeptidase N [EC 3.4.11.2] from *Escherichia coli* with a molecular weight of approximately 99,000 belongs to clan MA, family M1 and a zinc ion is found at its active center. It has been reported that this enzyme is the major aminopeptidase in *E. coli*, and it is involved in ATP-dependent downstream processing during cytosolic protein degradation (1). This enzyme is well conserved in a variety of species, such as mammals, insects, plants, and bacteria. Together with certain endopeptidases, aminopeptidase N plays a major role in peptide degradation, which enables the utilization of amino acids as nutrients. Additionally, aminopeptidase N is known to possess significant physiological functions as a receptor, irrespective of its enzymatic function. For example, human aminopeptidase N, which has been identified as a CD13 antigen, is known as a receptor for corona virus-like transmissible gastroenteritis virus and human corona virus 229E (2, 3). In addition, isoforms from *Bombbyx mori* have been reported to act as receptors for *Bacillus thuringensis* Cry1A toxin (4, 5). The human [gi:29840829] and *B. mori* [gi:3452557] enzymes, which are membrane-bound glycoproteins, unlike the *E. coli* counterpart, have a sequence identity of 13.6 and 13.4% with *E. coli* enzyme, respectively.

Aminopeptidase N was first purified from *E. coli* K-12 in 1982, and its catalytic properties were characterized using Ala-p-nitroanilide as a substrate by McCaman and Villarejo (6). The enzyme was described as a monomeric, acidic protein with a
molecular weight of 87,000, and it was reported to have a sulfhydryl group that was essential for its activity, since the activity of the enzyme was found to be inhibited by p-chloromercuribenzoate (PCMB). However, this activity was also later found to be inhibited by o-phenanthroline, puromycin, bestatin and amastatin; in another study, Yoshimoto et al. demonstrated that aminopeptidase N possessed an essential metal ion and a sulfydryl group involved in the catalytic activity of this enzyme (7). The nucleotide sequence of the pepN gene encoding aminopeptidase N was determined by Foglino and co-workers (8). This gene codes for a protein of 870 amino acid residues.

Although aminopeptidase N has been recognized since the 1980s, the three-dimensional structure of this enzyme had not yet been determined. Many aminopeptidases have been classified in the same family to date, including aminopeptidase A [EC 3.4.11.7], aminopeptidase B [EC 3.4.11.6], and oxytocinase [EC 3.4.11.3], and important biological functions have been attributed to these enzymes. However, structural information about this family of enzymes remains limited in fact, such information is currently available for only two such enzymes: leukotriene A₄ hydrolase [EC 3.3.2.6] from humans (9) and tricorn interacting factor F3 from Thermoplasma acidophilum (10). These enzymes show respective sequence identities of 15.7 and 15.3% with aminopeptidase N from E. coli. Leukotriene A₄ hydrolase is a very unique enzyme that exhibits both aminopeptidase activity as well as hydrolysis of leukotriene A₄. Although this enzyme differs markedly from other peptidases in this respect, the structural analysis of leukotriene A₄ hydrolase has demonstrated that the enzymes in this family possess a themolysin-like catalytic domain (9). Moreover, the structural analysis of tricorn interacting factor F3 revealed that two of four domains composing this enzyme, i.e., an N-terminal domain, which is composed of a β-sandwich structure, and a catalytic domain, are similar to those of leukotriene A₄ hydrolase. (10). Significant sequence conservation is recognized in the two domains among three enzymes, that of the aminopeptidase N has a sequence identity of 17.1% and 18.8% with leukotriene A₄ hydrolase and tricorn interacting factor F3, respectively. However the sequence identity of the C-terminal region with those of leukotriene A₄ hydrolase and tricorn interacting factor F3 is low, 12.8% and 13.0%, respectively. It was expected that the N-terminal and catalytic domains of aminopeptidase N may share a common folding pattern with that observed in this family of enzymes. The distinct structure of the C-terminal region is thought to be among those features that render these enzymes unique in terms of substrate specificity.

Namely, aminopeptidase N has broad specificity. This enzyme shows a hierarchical preference for substrates with N-terminal residues in the following order: Arg, Ala, and Lys. It is noteworthy that the enzyme shows a little activity against peptides with an N-terminal Pro residue (1, 7). It should be noted that this hierarchy of preferences for favorite N-terminal residues differs from that of the enzyme from human (11). In order to reveal catalytic and substrate-recognition mechanisms of aminopeptidase N, we cloned the gene encoding the enzyme, and expressed the enzyme in Escherichia coli. This is the first report of the crystal structure of aminopeptidase N, the catalytic and substrate recognition mechanisms of which are also discussed.

**EXPERIMENTAL PROCEDURES**

**Materials** – Ala-β-naphthylamide (Ala-βNA), Pro-βNA Fast Garnet GBC, and bestatin (N-[(2S,3R)-2-hydroxy-3-amino-4-phenylbutanoyl]-L-Leu) hydrochloride were purchased from Sigma Chemical Company. Ala-Phe-Pro-βNA was purchased from Bachem AG, Switzerland. DEAE-Toyopearl and Toyopearl HW65C were purchased from Tosoh Co., Tokyo, Japan. 2-(N-morpholino) ethanesulfonic acid (MES), and ammonium sulfate were purchased from Nacalai Tesque Inc., Kyoto, Japan. Gly-Pro-βNA was synthesized according to a previously described procedure (12). Prolyl aminopeptidase from Bacillus coagulans (13) and dipeptidyl aminopeptidase IV from...
Stenotrophomonas maltophilia (14) were purified as described previously.

**Bacterial Strains, Plasmids, and Media—** E. coli XL1-Blue (*recA1, endA1, gyrA96thi-1, hsdR17, supE44* were used for the expression, together with the plasmid pBluescript II SK(-)). The bacteria were grown in Luria-Bertani broth (LB-broth) and N-broth.

**Cloning of the pepN Gene—** The pepN gene was isolated from the Kohara clone 219. The phage DNA was digested with *PstI* and *XhoI*, and the 3.4-kb fragment containing the *pepN* open reading frame was cloned into the same restriction sites of pBluescript II SK(-) to produce pAN14. The nucleotide sequence of this clone was confirmed by DNA sequencing using the ABI Prism 3100 Avant Genetic Analyzer using the BigDye terminator v1.1 cycle sequencing kit.

**Purification of the Soluble Enzymes—** E. coli XL1-Blue were transformed with the recombinant plasmid pAN14, and the transformants were grown at 310 K in 200 ml of LB broth (50 μg ml⁻¹ ampicillin). Then, the transformants were aerobically cultured in 20 liters of N broth containing ampicillin (50 mg liter⁻¹) at 310 K for 17 hours in a jar fermenter (MBS). The harvested cells (40 g, wet weight) were washed with 20 mM Tris-HCl buffer (pH 7.0). The washed cells were resuspended in the same buffer and disrupted by repeated sonication on an ice bath using an Ultrasonic Disruptor (Tomy, UD200). To remove the nucleic acid, protamine sulfate (16 mg per wet weight) was added to the solution. After the cell debris was removed by centrifugation (22,250g; 20 min; 297 K), the supernatant was fractionated with ammonium sulfate from 35% to 80% saturation. The precipitate was dissolved in 20 mM Tris-HCl buffer (pH 7.0) containing 35% saturated ammonium sulfate, and the solution was applied to a Toyopearl HW65C (TOSOH) column equilibrated with the same buffer. The enzymes were eluted with a linear gradient from 35 to 0% saturation of ammonium sulfate. The eluted enzymes were dialyzed against 20 mM Tris-HCl buffer (pH 7.0), and the enzyme solution was applied to a DEAE-Toyopearl (TOSOH) column equilibrated with the same buffer. After elution with a linear gradient from 0 to 1 M sodium chloride was carried out, the active fractions were combined and dialyzed against 20 mM Tris-HCl buffer (pH 7.0). The purity of the isolated enzyme was confirmed by SDS-PAGE. The purified enzyme was concentrated to 28.0 mg ml⁻¹ using a Centriprep YM-30 device (Millipore).

**Enzyme Activity Assay—** The enzyme activity was assayed using Ala-βNA as the substrate. The reaction mixture consisted of 0.8 ml of 20 mM Tris-HCl (pH 7.0), 0.1 ml of enzyme solution, and 0.1 ml of a 3 mM solution of substrate. After a 10-min incubation at 310 K, the reaction was stopped by the addition of 0.5 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 20 min. One unit of activity was defined as the amount of enzyme that released 1 μmol of β-naphthylamine per min under the above conditions. To determine the *K_m* values of the aminopeptidase N against Ala-βNA and Pro-βNA, the concentration of the substrate was varied. Lineweaver-Burk plots were used to calculate *K_m* and the apparent *V_max*. The enzyme concentration was estimated based on a molar absorbance coefficient of 113,650, and a molecular weight of 98,918 was used for the *k_cat* calculations.

The reactivity of mixed enzyme solutions was measured in a similar manner. The following enzyme solutions were used in this series: aminopeptidase N, prolyl aminopeptidase from *B. coagulans*, and dipeptidyl aminopeptidase IV from *S. maltophilia*. The latter two enzymes were used alone or were mixed with aminopeptidase N for an assay using three substrates, Pro-βNA, Gly-Pro-βNA, and Ala-Phe-Pro-βNA. In the case of the assay using a mixture of two enzymes, the reaction mixture consisted of 0.7 ml of 20 mM Tris-HCl buffer (pH 7.0), 0.1 ml of aminopeptidase N, 0.1 ml of the second enzyme solution, and 0.1 ml of a 3 mM solution of substrate. For all activity measurements, the concentration of each enzyme was kept constant, i.e., 0.804 mg ml⁻¹ of aminopeptidase N, 0.100 μg ml⁻¹ of prolyl aminopeptidase, and 3.21 μg ml⁻¹ of dipeptidyl aminopeptidase IV were used.
Crystallization and Data Collection – Crystallization was carried out at 293 K by the hanging-drop vapor-diffusion method using ammonium sulfate as a precipitant. A droplet was mixed with 28.0 mg ml\(^{-1}\) protein solution with the same amount of reservoir solution, and the sample was equilibrated against the reservoir solution (1.75 M ammonium sulfate, 100 mM MES buffer (pH 6.4)). After 5 days, prism crystals grew to dimensions of 0.3 x 0.3 x 0.3 mm, and they belonged to the space group \(P3_12_1\) with the following cell dimensions: \(a = b = 120.5\), \(c = 170.8\) Å, \(\gamma = 120^\circ\). One molecule was found in an asymmetric unit, and 66% of the crystal volume was occupied by solvent. Crystals of the enzyme-bestatin complex were obtained by soaking ligand-free crystals for 48 hours in prepared solution that contained 1 mM bestatin, 2.2 M ammonium sulfate, and 100 mM MES buffer (pH 6.4). The diffraction data for the ligand-free form and the enzyme-bestatin complex were collected to 1.50 and 1.60 Å resolution respectively, at 100 K using a wavelength of 1.0000 Å from the synchrotron radiation source at the Photon Factory AR-NW12 station (Tsukuba, Japan). For data collection under cryogenic conditions, the crystals were soaked for one minute in a prepared solution containing 30%(v/v) glycerol, 1.75 M ammonium sulfate, and 100 mM MES buffer (pH 6.4). The crystals were mounted in a nylon loop and flash-cooled at 100 K. The data sets for crystals soaked in 0.1 mM ethylmercurithiosalicylate (EMTS) for 12 hours and 0.1 mM methyl mercury chloride for 21 hours were respectively collected to 2.1 and 2.0 Å resolution at 100 K with a Rigaku R-Axis IV\(^{++}\) detector using CuK\(\alpha\) radiation, which was generated by a Rigaku MicroMax007 rotating-anode X-ray generator with osmic confocal focusing mirrors. The data set for the crystal soaked in 1 mM \(p\)-chloromercuribenzoate (PCMB) for 1 day was collected to 1.8 Å resolution at 100 K at the Photon Factory AR-NW12 station. The data for the ligand-free form and the enzyme-bestatin complex were processed and scaled by HKL2000 (15), and the heavy atom derivative data were processed using MOSFLM (16) and SCALA from the CCP4 suite (17) (Table I).

Structure Determination and Refinement – The phase problem was solved by the multiple isomorphous replacement method (MIR) using three and two mercury sites from EMTS and MeHgCl derivatives, respectively. The scaling of all data and map calculations were performed with the CCP4 program suite (17). The determination of mercury sites, the refinement of the heavy atom parameters, and the calculations of the initial phases were performed using the program SOLVE (18), resulting in a mean figures of merit of 0.60 at 2.1 Å resolution. The map was improved by the process of solvent flattening with the program RESOLVE (19), to give a mean figure of 0.77 at 2.1 Å resolution. The initial model was automatically built using the program ARP/wARP (20), and the model was manually complemented with the program XtalView (21). The structure of the ligand-free form was refined by simulated annealing and energy minimization with the program CNS (22) using the data obtained from 30 to 1.50 Å resolution. The structure was examined by inspecting the composite omit map. The refinement and model rebuilding were alternately carried out using several cycles, and then water molecules were selected on the basis of the peak height and the distance criteria from the difference map. The water molecules whose thermal factors exceeded 50 Å\(^2\) after refinement were removed from the list. Further model building and refinement cycles gave an \(R\) factor of 18.1% and \(R_{\text{free}}\) of 19.1% using 228,266 reflections from 20 to 1.5 Å resolution. The maximal thermal factor of the water molecules was 50 Å\(^2\).

The same refinement procedure was applied to the enzyme-bestatin complex, with the exception that the coordinates of the ligand-free form were used for the initial model. After a refinement cycle involving simulated annealing and energy minimization, the difference Fourier map obviously displayed the residual electron density corresponding to the bestatin bound at the active site. Water molecules were selected from the difference map, and further model building and refinement cycles were carried out, resulting in an \(R\) factor of 18.2% and \(R_{\text{free}}\) of 19.4% using 186,911 reflections from 20 to 1.6 Å resolution, with a maximal thermal factor of water molecules of 41 Å\(^2\).
RESULTS

Construction of Expression Plasmid and Purification of the Enzyme – Since high activity was confirmed in the soluble fraction of the extracts of E. coli XL1-Blue transformed with pAN14, it was used for production of the enzyme. After cultivation of the transformant in 20 liters of N-broth in a jar fermenter, aminopeptidase N was purified following sequential chromatography on Toyopearl HW65C and DEAE-Toyopearl columns. The enzyme was purified with a specific activity of 43.8-fold and a recovery of 40%.

Activity toward Proline-containing Substrates – The kinetic parameters of aminopeptidase N against Ala-βNA and Pro-βNA are shown in Table II. The $k_{cat}/K_M$ value against Pro-βNA was approximately 0.38% of that against Ala-βNA. Although the reactivity of aminopeptidase N against Pro-βNA was very low, the enzyme was able to cleave the peptide bond of Pro-Xaa. However, neither Gly-Pro-βNA nor Ala-Phe-Pro-βNA was hydrolyzed to release β-naphthylamine, even after prolonged incubation time (Table III). Prolyl aminopeptidase and dipeptidyl aminopeptidase IV are serine peptidases, which specifically release Pro and Xaa-Pro from the substrates, respectively (13, 14). A mixed solution of the aminopeptidase N and prolyl aminopeptidase showed activity against only Pro-βNA among the three substrates. In contrast, the enzyme mixture of aminopeptidase N and dipeptidyl aminopeptidase IV degraded all three substrates. These results indicated that the peptide bond of Xaa-Pro was not hydrolyzed by aminopeptidase N.

Quality of the Structure – The refined ligand-free model contains 865 residues, a zinc ion, a sulfate anion, and 1127 water molecules with an $R_{factor}$ of 18.1% at a 1.5 Å resolution. The model contained Ala122 of the cis-peptide. On the basis of the electron density map, the conformation of the Ala122 residue was confirmed. This model lacked the five N-terminal residues, due to a lack of interpretable electron density. The average thermal factor of the main-chain atoms, the side-chain atoms, and the water molecules was 14.3, 19.9, and 28.9 Å², respectively. Analysis of the stereochemistry with PROCHECK (23) demonstrated that all of the main chain atoms fell within the additional allowed region of the Ramachandran plot, with 734 residues (93.0%) in the most favored region, and 55 residues (7.0%) in the additional allowed region. The refined model of the enzyme-bestatin complex contained 867 residues without the three N-terminal residues; a bestatin; a zinc ion; a sulfate anion; and 946 water molecules with an $R_{factor}$ of 18.2% at a 1.6 Å resolution. The average thermal factor of the main-chain atoms, side-chain atoms, bestatin, and water molecules was 13.8, 18.6, 19.6, and 25.6 Å², respectively. Based on the PROCHECK analysis, 735 residues fell within the most favored region, and 55 residues within the additional allowed region of the Ramachandran plot.

Overall Structure and Subunit Assembly – The overall structure and surface of aminopeptidase N is shown in Fig. 1a and 1b. Aminopeptidase N has dimensions of approximately 85 Å x 60 Å x 45 Å, and an accessible surface area (ASA) of 32,749 Å², as calculated using AREAIMOL from the CCP4 suite (17). The $C\alpha$ positions of the ligand-free form were superimposed onto those of the enzyme-bestatin complex with an rms (root-mean-square) deviation of 0.09 Å, indicating that both structures were identical. The structure of aminopeptidase N consists of twenty-six α-helices and twenty-six β-strands, and can be divided into four domains, i.e., an N-terminal β-domain (Gln6-Asp193), a catalytic domain (Phe194-Gly444), a middle β-domain (Thr445-Trp546), and a C-terminal α-domain (Ser547-Ala870). The first three domains, in sequence, are connected in a linear fashion, and the last domain is located at the side of the catalytic domain, serving as a cover for this domain. Consequently, the catalytic domain interacts with all other domains, and the domain-interface area of the catalytic domain, calculated on the basis of the ASA, is 741 Å² (with the N-terminal β-domain), 423 Å² (with the middle β-domain), and 852 Å² (with the C-terminal α-domain), respectively. The domain-interface area between the
middle β-domain and the C-terminal α-domain was found to be 298 Å². Although the N-terminal β-domain primarily interacts with the catalytic domain, a significant interaction with the C-terminal α-domain was observed with a domain-interface of 112 Å². The hydrophobic interaction was found to be predominant on all domain interfaces.

The N-terminal β-domain forms a β-sandwich structure composed of three β-sheets. The seven-stranded β-sheet (β12β13β10β1β2β7β4) is located on the opposite side of the three-stranded (β3β5β8) and the four-stranded (β11β14β9β8) antiparallel β-sheets. The seven- and three-stranded β-sheets were exposed to the solvent area, and the four-stranded β-sheet was found to be predominantly involved in the domain interface with a β-sheet of the catalytic domain. The catalytic domain has an α/β-structure formed by a five-stranded β-sheet and eight α-helices, and this domain is similar to thermolysin, an endopeptidase. The catalytic domain was superimposed on Bacillus thermoproteolyticus thermolysin (PDB code 4tln; 24), with an rms deviation of 1.78 Å for the corresponding 191 Cα positions. The middle β-domain is comprised of two β-sheets (β24β21β20β26 and β23β22β25), and forms a β-sandwich structure. The structure of this domain is unique, and the role this domain plays within organisms remains unclear. The C-terminal α-domain is composed of eighteen helices (α9-α26), and these helices are arranged in an anti-parallel manner. The C-terminal α-domain has two layers of parallel α-helices: eight in the inner layer (α10, α12, α16, α18, α20, α22, α24, and α26) and eight in the outer layer (α9, α11, α15, α17, α19, α21, α23, and α25). These helices form an arched helical bundle, and then two helices (α13α14) are positioned such that they block the arched entrance. Consequently, the C-terminal α-domain has a hole at the center, as shown in Fig 1b, the diameter of which is ca. 8Å.

Aminopeptidase N has a metal binding motif of HEXXHx5E in the catalytic domain, which is well conserved in the peptidase family M1 (25). There are two histidine residues from the α2 helix, and a third ligating glutamate residue from the α3 helix. The active site is found in a groove that consists of one β-strand and three α-helices; the β18 strand and the α5 helix form each side wall, and α2 and α3 helices serve as the base. The groove on the catalytic domain is shown in Fig. 1c. One end of this groove is closed by the N-terminal β-domain, and the other end and the top of the groove are covered by the C-terminal α-domain. As a result, a large cavity with an entrance at the center of the C-terminal domain is formed by three domains inside the protein molecule.

**Active site of Aminopeptidase N** – A ribbon model and schematic diagrams of the active site in the ligand-free form are shown in Fig. 2a and 2b. In aminopeptidase N from *E. coli*, His297, Glu298, His301, and Glu320 form the zinc binding motif (HEXXHx5E). The zinc ion is coordinated by the Nε atoms of His297 and His301, the carboxyl Oɛ atom of Glu320, and a water molecule (Wat1). These four atoms give a tetrahedral coordination with a central zinc ion. The Wat1 molecule enables hydrogen binding to Glu298. It is expected that the Wat1 molecule is the nucleophile that attacks the carbonyl carbon of the substrate, and the Glu298 functions as a catalytic base for peptide cleavage, similar to its function in other zinc proteases (25, 26).

The Glu298 residue is surrounded by Ala262, the main chain of Met263, the side chains of Phe271 and Val294, and the Cδ atoms of the two zinc-coordinated histidines.

The Gly-Ala-Met-Glu-Asn (GAMEN) sequence, which includes the abovementioned residues Ala262 and Met263, is also well conserved in aminopeptidase N. The Glu264 residue corresponds to the Glu355 residue in the human enzyme, and this latter residue is reportedly involved in recognizing the N-terminal amino group on substrates (27). The Glu264 residue forms a hydrogen bond with a water molecule (Wat2). The Wat2 molecule interacts with the Glu121 residue, which is located in the N-terminal β-domain. The Glu121 and Glu264 residues form hydrogen bonds to the Lys319 residue. The Lys319 residue can form hydrogen bonds to the Glu320 residue, and Glu320 is among the zinc ligands for this enzyme.
Enzyme Structure Complexed with Bestatin – Diagrams of the active site in the enzyme-bestatin complex are shown in Fig. 2c and 2d. Bestatin is an inhibitor that imitates a dipeptide. The bestatin is located in the groove of the active site. The electron density corresponding to bestatin was well defined, as shown in Fig. 3. In the enzyme-bestatin complex, the N-terminal amino group of the bestatin forms hydrogen bonds to Glu121 and Glu264. A 2-hydroxy group and the 1-carbonyl oxygen of bestatin are coordinated to the zinc ion. The 2-hydroxy group and the 1-carbonyl oxygen form hydrogen bonds with Glu298 and Tyr381 residues. The superpositioning of the ligand-free form and the enzyme-bestatin complex revealed that the positions of Wat1 and Wat2 in the ligand-free form revealed a virtually complete overlap with the molecules of the amino group and the hydroxyl group of bestatin, respectively. The positions of almost all of the residues at the active site coincided in the two structures. However, it should be noted that the side chain of Met260 alone gave a different conformation.

DISCUSSION

Comparison of Structures in Peptidase Family M1 – Aminopeptidase N from E. coli is composed of four domains. The overall structure of aminopeptidase N is similar to that of two enzymes belonging to the peptidase M1 family (Fig. 4). However, there are distinct structural characteristics in E. coli aminopeptidase N. The structures of leukotriene A₄ hydrolyase (PDB code 1hs6; 9) and tricorn interacting factor F3 (PDB code 1z1w and 1z5h; 10) were superposed onto that of aminopeptidase N. This superpositioning demonstrated a high degree of similarity among these three enzymes, especially in terms of the respective structures of the N-terminal β-domain and the catalytic domain, in which the rms deviations were calculated to be 2.36 and 2.07 Å, respectively (Fig. 5). This finding was consistent with the sequence alignment of these enzymes. However, in contrast to the structures of the two N-terminal domains, those of the middle β-domain and the C-terminal α-domain differed significantly from each other. In the case of leukotriene A₄ hydrolyase, no domain corresponds to the middle β-domain of aminopeptidase N, and the C-terminal α-domain that consists of eight α-helices is clearly smaller than that of aminopeptidase N. Tricorn interacting factor F3 has a middle β-domain and a C-terminal α-domain that consists of sixteen α-helices. Aminopeptidase N has the largest C-terminal α-domain among these enzymes, which consists of eighteen α-helices. In all three enzymes, folding of the region from the α₁₅ to the α₂₂–helix, which corresponds to the C-terminal α-domain of leukotriene A₄ hydrolase, is common. A key difference in the C-terminal α-domain between aminopeptidase N and tricorn interacting factor F3 is the presence of a central hole in aminopeptidase N. This hole is produced by two helices, α₁₃ and α₁₄, which are not present in the domain of tricorn interacting factor F3. Furthermore, the C-terminal domains of these latter two enzymes differ in terms of their relative position with respect to other domains. The domain of tricorn interacting factor F3 does not interact with the N-terminal β-domain, and the area that makes contact with the catalytic domain is narrower than that of aminopeptidase N.

The active center of the three enzymes is located in a groove that consists of one β-strand and three α-helices on a thermolysin-like catalytic domain. One end of each groove is covered by the N-terminal β-domain, and the other end and the top of the groove are covered by the C-terminal α-domain. Figure 6 shows the protein surface diagrams of the three enzymes. Due to the differences in the shape of the C-terminal α-domain and the relative position of this domain with respect to the catalytic domain, different substrate routes of entry to the individual active sites could be estimated, as indicated by the arrows in the figure. Leukotriene A₄ hydrolase has a deep tunnel that reaches the active center from the protein surface. As tricorn interacting factor F3, which forms a hook-like structure (10), has a deep and wide cleft, its active site is largely exposed to solvent area. In the case of aminopeptidase N, the C-terminal domain with a small hole completely covers the groove on the catalytic domain. As a result, a very
large cavity is formed inside the protein. In this case, a substrate could enter the cavity via the tunnel at the C-terminal α-domain, although the diameter of the entrance may not be sufficient to allow for the passage of a lengthy peptide. It has been proposed that tricorn interacting factor F3 undergoes a conformational change in the C-terminal domain due to its flexibility (10). It is possible that aminopeptidase N forms a hook-like structure in a manner much like that of tricorn interacting factor F3. However, the conformation of the C-terminal α-domain in the crystal did not appear to change due to interaction with a neighboring molecule. In fact, the enzyme-bestatin complex was obtained simply by soaking the crystal in a solution containing bestatin, and the structure was virtually identical to that of the ligand-free form. Consequently, it appears more likely that at least small peptides and inhibitors (e.g., bestatin) are able to enter the active site via the small hole, without requiring any substantial conformational changes in the overall structure. Since aminopeptidase N exhibits hydrolytic activity against a broad range of substrates, it is possible that the overall structure of the enzyme could be involved in restraining substrates in an organism, e.g., by limiting the peptide length of the substrate. In order to reveal the pathway for the entry of substrates to the active site, we are planning a future investigation of site-directed mutant enzymes using kinetic and X-ray crystallographic approaches.

**Substrate Recognition of Aminopeptidase N from E. coli** – In a study of human aminopeptidase N, Luciani and colleagues revealed that the Glu355 residue is involved in binding to the N-terminal amino group of substrates (27). In the enzyme from *E. coli*, the residue that corresponds to Glu355 is Glu264. The structure of aminopeptidase N complexed with bestatin revealed that the N-terminal binding site contains two Glu residues, Glu121 and Glu264. At the active site of the ligand-free form, the Wat2 molecule forms hydrogen bonds with these Glu121 and Glu264 residues. The position of the Wat2 molecule virtually overlaps that of the amino group of bestatin. Although the N-terminal residue of bestatin imitates d-Phe, the amino group is expected to be located at the same position of a substrate containing an L-amino acid. Additionally, it is quite likely that the phenyl group of bestatin occupies the same space that would accommodate the N-terminal side-chain of a substrate. Comparison of the ligand-free form with the enzyme-bestatin complex revealed that the most noticeable difference was a conformational change in the side chain of Met260. Namely, the cavity of the S1 site is occupied by the side chain of Met260 in the ligand-free form, thus rendering the size of cavity suitable for the accommodation of Ala or Pro residues. In the enzyme-bestatin complex, the side chain shifts to enlarge the S1 site, such that a phenyl group would be able to enter the cavity. The Met260 residue is expected to function as a cushion for substrates with amino terminal residues of different sizes; such changes in conformation would in turn alter the size of the pocket. In the human and *B. mori* enzymes, the methionine residue is not conserved, and the residues corresponding to Met260 are Ala351 and Ala324, respectively. However, the methionine residue is well conserved in the forms of the enzyme from proteobacteria such as *Salmonella typhimurium* [gi:16759938], *Erwinia carotovora* [gi:50121463], *Yersinia pestis KIM* [gi:22126634], *Shigella flexneri* [gi:24112341], *Pasteurella multocida* [gi:15602483], *Mannheimia succiniciproducens* [gi:52425089], and *Pseudomonas aeruginosa* [gi:15598279], etc. It is expected that the conserved Met260 residue in the bacterial enzymes would exert a strong effect on the preferential substrates for aminopeptidase N. Aminopeptidase N is known to degrade Pro-βNA. However, the structure of the N-terminal binding site suggests that an N-terminal Pro residue on a substrate would have difficulty binding to the S1 site, due to steric hindrance between Glu121 and the pyrrolidine ring. Nonetheless, the conformation of Met260 in the ligand-free form appears appropriate for the size of the S1 site, i.e., that of Pro residue. The flexible Met260 residue may contribute to the binding of a Pro residue at the P1 site via its hydrophobic interaction with the pyrrolidine ring. Further studies will be needed to elucidate the function of Met260.
Taken together, the abovementioned findings suggested the building of Michaelis complex models with L-Ala-L-Leu or L-Arg-L-Leu, as shown in Fig. 7. The conformation with the Met260 residue in the ligand-free form and the enzyme-bestatin complex was adopted as it was in the complex models containing L-Ala-L-Leu and L-Arg-L-Leu, respectively. The N-terminal amino group of the substrate forms hydrogen bonds to the carboxyl groups of Glu121 and Glu264. Then, the carbonyl oxygen at the P1 position coordinates with the zinc ion. Thus it can be deduced that the N atom at the P1' position forms hydrogen bonds with Glu298 and the main-chain carbonyl oxygen of Ala262.

Aminopeptidase N exhibits broad specificity; it is even able to hydrolyze peptide bonds in Pro-Xaa. However, aminopeptidase N does not hydrolyze the peptide bond in Xaa-Pro. Our proposed models clearly account for why aminopeptidase N is unable to cleave the peptide bond of Xaa-Pro. When the five-coordinate intermediate is formed following substrate binding, the pyrrolidine ring of Pro at the P1' site does not fit into the groove as does the Leu residue shown in Fig. 7, and therefore the ring would be forced to situate itself perpendicular to the groove to account for the specific conformation of proline. Consequently, it is likely that due to the steric hindrance between the pyrrolidine ring and the main-chain of Ala262, this conformation of proline would preclude binding to the active site. Alternatively, if the Pro residue were to be accommodated within the groove, then the carbonyl oxygen at P1 position would not bind to the zinc ion. Accordingly, the hydrolysis reaction would not proceed any further.

It is of note that the enzyme prefers substrates with an Arg residue at the N-terminal position. As shown in Fig. 7b, when such a substrate is bound to the active site, the guanidino group of arginine can form hydrogen bonds with carbonyl oxygen of Asn373 and Gln821 residues, which respectively belong to the catalytic domain and the C-terminal α-domain. These residues are located at the top of the pocket in order to accommodate the N-terminal side chain of substrates.

The carbonyl oxygen of these residues are respectively located at distances of 9.19 and 9.63 Å from the N-terminal Ca position of bestatin; these distances are sufficient for the formation of hydrogen bonds with the side chain of arginine. Human aminopeptidase N prefers substrates with Ala or Lys residues at the N-terminus over those with an Arg residue, and its lowest $K_M$ value has been reported to be against Lys-βNA (11). It is likely that this preference is a result of differences in component residues at the corresponding position of the active site. The sequence alignment suggests that the corresponding residues are Glu467 and Thr911 residues, respectively. These residues are expected be related to the Lys preference of the human enzyme. It will be necessary to clarify the roles played by these residues in future studies involving site-directed mutagenesis and other approaches.

Inhibition of the enzyme with mercury reagents – Since aminopeptidase N can be inhibited by mercuric reagents such as PCMB, the enzyme has been reported to possess a reactive sulfhydryl group in addition to the metal ion (6, 7). Aminopeptidase N contains eight cysteine residues: Cys117, Cys120, Cys184, Cys310, Cys418, Cys534, Cys684, and Cys727. However, none of these Cys residues is exposed to the surface of the protein. The difference Fourier map calculated using the data of the PCMB derivative revealed four peaks corresponding to mercury. One of these peaks displayed the residual electron density of PCMB. Covalently bound mercury sites were only found at Cys418, Cys534, and Cys727, and another mercury atom interacted with the Ser701 and His705 residues; all residues interacting with mercury were located in the exterior surface of the protein. There were no peaks corresponding to mercury, i.e., none were observed either at the active site or at any other sites. The crystals of the PCMB derivative prepared by the soaking method showed activity against Ala-βNA upon measurements made after back-soaking the crystal in heavy atom-free mother liquor (data not shown). The proteins in the crystal are generally restrained from undergoing large conformational changes, thus
indicating that the inhibition by a mercury reagent was due to Cys binding or binding to other residues hidden in the protein. For instance, Cys120 and Cys310 are located near the active site, as well as near the domain interface between the N-terminal β- and catalytic domains. Therefore, it is possible that protein folding and the structure of the active site were distorted by the binding of the mercury reagent to these residues.

Implication of Catalytic Mechanism – The catalytic mechanism of aminopeptidase N is proposed as shown in Fig. 8. Wat1, which coordinates to the zinc ion, is a nucleophilic agent. The nucleophilicity of Wat1 is strongly activated by the zinc ion and by hydrogen bonding to the Glu298 residue. The hydrolysis reaction is initiated by a nucleophilic attack at the carbonyl carbon of the substrate by Wat1 after the formation of a Michaelis complex. The Glu298 residue serves as a general base, as has also been proposed in the case of thermolysin and other enzymes from the same super family (10, 28-29). It is expected that the Glu298 residue is exposed to a hydrophobic environment upon substrate binding. The Glu298 residue is surrounded by Phe271 and Val294. These hydrophobic residues can be found in related enzymes from other organisms, e.g., Tyr362 and Val385 in humans, and Tyr332 and Ile355 in Bomyx mori. The hydrophobic environment around Glu298 appears to be important for the activation of the residue as a catalytic base.

The Tyr381 residue is located near Glu320 and a zinc ion. In the enzyme-bestatin complex, the Tyr381 residue forms a hydrogen bond with the 1-carbonyl oxygen of bestatin. The Tyr381 residue is well conserved in peptidase family M1; the corresponding residues are Tyr383 and Tyr351 in leukotriene A4 hydrolase and tricorn interacting factor F3, respectively, although the conformation of Tyr351 residue differs from that of the corresponding residue in aminopeptidase N. It has been proposed that the Tyr351 residue is related to the catalytic mechanism via this conformational change (10). These tyrosine residues are located at a position similar to that of the His231 residue from thermolysin (24), and the tyrosine residue is believed to carry out the same function as the His231 residue (9, 10). Leukotriene A4 hydrolase exhibits not only hydrolase activity, but also aminopeptidase activity, and a bifunctional mechanism has been proposed (9, 28). The proposed mechanism of aminopeptidase activity was initially based on that of thermolysin, as derived from a computer graphics study (26). The findings suggested that the Tyr381 residue, which forms a hydrogen bond with the nitrogen atom at the P1’ position of the substrate, acts as a proton donor in the final step of the hydrolysis reaction, and this residue functions in a manner similar to that of His231 of thermolysin (28). However, a theoretical study of the catalytic mechanism of thermolysin by Pelmenschikov and collaborators suggested that the interaction between the carbonyl anion and the His231-Asp226 linkage allows for a decrease in the activation energy (30). In the case of aminopeptidase N, the distance between the hydroxyl group of the Tyr381 residue and the expected S1’ nitrogen would be too great to enable any interaction. Our models indicate that the Tyr381 residue forms a hydrogen bond with the carbonyl oxygen at the P1 position of the substrate. As a consequence, it is thought that the Tyr381 residue is located at a position at which the hydroxyl group interacts with the carbonyl anion from the reaction intermediate. Accordingly, the Tyr381 residue is thought to contribute to the stabilization of the reaction intermediate together with the zinc ion, much like the putative role of the His231 residue proposed by Pelmenschikov and colleagues. The serine proteases usually possess an oxyanion hole for stabilization of the reaction intermediate. It is generally known that the amide nitrogen of the main chain plays a role as an oxyanion hole. In addition, it has been reported that prolyl oligopeptidase and dipeptidyl peptidase IV, both of which belong to family S9, possess a tyrosine residue that acts as the oxyanion hole. (31-34). It is likely that the tyrosine residues in family M1 exert a function similar to that of forming an oxyanion hole in serine proteases. To elucidate the role of the Tyr381 residue, we plan to study a model of the intermediate state, i.e., the enzyme complexed with substrate analogues, using X-ray crystallography.
The Glu298 residue is thought to transfer the proton received from the Wat1 molecule to the amide nitrogen from the P1' position, and the product would then be released. Finally, the cleaved N-terminal residue bound to the zinc ion is expected to be exchanged with a water molecule and released. It is thus proposed that the catalytic reaction of aminopeptidase N proceeds by the mechanism described here.

REFERENCES


FOOTNOTES

The atomic coordinates and structure factors for ligand-free enzyme and enzyme complexed with a bestatin (PDB code 2DQ6 and 2DQM) have been deposited in the Worldwide Protein Data Bank (wwPDB; http://www.wwpdb.org), the Protein Data Bank Japan at the Institute for Protein Research in Osaka University (PDBj; http://www.pdbj.org/)

*This work was supported in part by the ‘National Project on Protein Structural and Functional Analyses’ run by the Japanese Ministry of Education, Culture, Sports, Science and Technology.

†These authors contributed equally to this work.
‡Present address: College of Pharmaceutical Sciences, Daiichi University, Fukuoka 815-8511, Japan

The abbreviations used are: APN, aminopeptidase N; ASA, accessible surface area; -βNA, -β-naphthylamide; DALI, Distance matrix alignment; DPIV, dipeptidyl aminopeptidase IV; EDTA, ethylenediaminetetraacetic acid; EMTS, ethyl mercury thiosalicylate; IPTG, isopropyl 1-thio-β-D-galactoside; LTA4H, leukotriene A₄ hydrolase; MES, 2-(N-morpholino) ethanesulfonic acid; MIR, multiple isomorphous replacement; PAP, prolyl aminopeptidase; rms, root-mean-square; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TIFF3, tricorn interacting factor F3.

FIGURE LEGENDS

Fig. 1 Overall structure of aminopeptidase N
(a) Stereo diagram of a ribbon model. The N-terminal β-, catalytic, middle β-, and C-terminal α-domains.
are represented by blue, cyan, green, and pink, respectively. The diagram was produced with the program POVS
script \(^{(35)}\) and rendered with POV-Ray (http://www.povray.org). (b) A diagram of the protein surface colored by each domain. The C-terminal \(\alpha\)-domain has a hole at the center of the domain. (c) A diagram of the protein surface without the C-terminal \(\alpha\)-domain. The area of contact between the C-terminal \(\alpha\)-domain and the other domains is represented in black. The active center of the enzyme, the zinc ion of which is indicated in magenta, is located at the groove on the catalytic domain, and this groove is covered by the dome-shaped C-terminal \(\alpha\)-domain. Consequently, the active site of aminopeptidase N is located within the protein. The surface diagrams were rendered with PyMol (http://www.pymol.org).

Fig. 2 The active site of aminopeptidase N.

The active site in the ligand-free form is shown as a ribbon-and-stick model (a), and as a scheme diagram (b). A zinc ion (pink) exhibits four-coordinated by His297, and His301, Glu320, and Wat1. Wat1 act as a nucleophilic agent, and Glu298 functions as a catalytic base. The active site in the enzyme-bestatin complex is shown as a ribbon-and-stick model (c), and a scheme diagram (d). The bestatin at the active site is shown in red. A zinc ion in the complex is five-coordinated by His297, His301, Glu320, and two oxygen atoms of the bestatin. The ribbon models were drawn with POVS
script \(^{(35)}\) and POV-ray (http://www.povray.org).

Fig. 3 A Stereo view of bestatin binding to the active site of aminopeptidase N

Bestatin is shown as a ball-and-stick model with an \(Fo-Fc\) omit map contoured at 3.5 \(\sigma\) levels. The difference Fourier map was calculated using the program CNS (21) without consideration of the structure factor of bestatin. The zinc ion is represented as a pink sphere, and residues at the active site are shown as stick models.

Fig. 4 Structure-based sequence alignment of aminopeptidase N (APN) with human leukotriene A\(_4\) hydrolase (LTA4H, PDB code 1hs6) and \(T.\) acidophilum tricorn interacting factor F3 (TIFF3, PDB code 1z1w).

The primary structures are aligned on the basis of the three-dimensional structures obtained using DALI (36). The N-terminal residues for which no electron density was observed are decapitalized. The secondary structural elements are indicated by boxes showing the \(\alpha\)-helices, and arrows showing the \(\beta\)-strands. These elements were assigned by the program DSSP (37). The numbering of the secondary structure was based on that for aminopeptidase N. The well-conserved sequences (GAMEN and HEXXHX\(_{18}\)E) among enzymes from this family are surrounded by boxes. The marks in the diagram indicate the respective glutamate residues binding to N-terminal of substrates (asterisk, *); a catalytic base (dagger, †); a tyrosine residue expected to stabilize the reaction intermediate (dabble dagger, ‡); hydrophobic residues around Glu298 (hash, #); and zinc-coordinated residues (pilcrow, ¶).

Fig. 5 C\(_{\alpha}\) trace stereo diagram of superimposed family enzymes.

The four domains of aminopeptidase N are shown in blue (N-terminal \(\beta\)-), cyan (catalytic), green (middle \(\beta\)-), and pink (C-terminal \(\alpha\)-). Leukotriene A\(_4\) hydrolase (1hs6) and tricorn interacting factor F3 (1z1w) are indicated by black and dashed lines, respectively. The structures of other enzymes in this family are superimposed onto that of ligand-free aminopeptidase N using a least-square method based on the corresponding C\(_{\alpha}\) positions. In these three enzymes, the structures of the N-terminal \(\beta\)- and catalytic domains are very similar to each other. However, the structures of the middle \(\beta\)- and C-terminal
α-domains differed among these enzymes. This diagram was created with the program MolScript (38).

**Fig. 6** Protein surface stereo diagrams of peptidase family M1 enzymes.

Three diagrams that respectively show aminopeptidase N (a), human leukotriene A₄ hydrolase (b, 1hs6), and *T. acidophilum* tricorn interacting factor F3 (c, 1z1w). The three enzymes are indicated in the respective colors used to represent corresponding regions, as based on the domains of aminopeptidase N, i.e., N-terminal β- (blue), catalytic (cyan), middle β- (green), and C-terminal α- (pink) domains. The arrows indicate the expected site for the entry of substrates into the active site of the three enzymes.

**Fig. 7** Expected substrate binding models of aminopeptidase N.

(a) The model of L-Ala-L-Leu binding to the ligand-free form. (b) The model of L-Arg-L-Leu binding to the protein structure from the enzyme-bestatin complex. The images were produced with the program PyMol (www.pymol.org). Scheme diagrams of expected interactions of the N-terminal residue, L-Ala (c), and L-Arg (d) with residues of the active site when the Michaelis complex is formed. The protein and residues in the active site are indicated as a ribbon model and a ball-and-stick model using CPK colors, respectively. Stick models of the substrates are shown in blue, which represents carbon atoms. A surface section of the active site is displayed in a color-coded manner on a van der Waals surface calculated using APBS (39), where red and blue represent net negative and positive charges, and white represents generally neutral positions, respectively. The conformation of Met260 from the ligand-free form gives a pocket suitable for the accommodation of the N-terminal alanine residue of the substrate. In contrast, Met260 from the enzyme-bestatin complex is located such that the pocket is enlarged.

**Fig. 8** Catalytic mechanism of aminopeptidase N.

(a) Free enzyme. (b) Michaelis complex. (c) Tetrahedral intermediate. (d) Enzyme complexed with an amino acid product
Table I Data collection, MIR and refinement statistics.

<table>
<thead>
<tr>
<th></th>
<th>Ligand-free</th>
<th>Bestatin complex</th>
<th>EMTS</th>
<th>MeHgCl</th>
<th>PCMB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P3(^{21})</td>
<td>P3(^{21})</td>
<td>P3(^{21})</td>
<td>P3(^{21})</td>
<td>P3(^{21})</td>
</tr>
<tr>
<td>Cell parameter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a, b) (Å)</td>
<td>120.5</td>
<td>120.4</td>
<td>120.6</td>
<td>120.4</td>
<td>120.6</td>
</tr>
<tr>
<td>(c) (Å)</td>
<td>170.8</td>
<td>170.9</td>
<td>170.8</td>
<td>170.8</td>
<td>170.9</td>
</tr>
<tr>
<td>Wavelength</td>
<td>1.000</td>
<td>1.000</td>
<td>1.5418</td>
<td>1.5418</td>
<td>1.000</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>50-1.50(1.55-1.50)</td>
<td>50-1.60(1.69-1.60)</td>
<td>50-2.10(2.21-2.10)</td>
<td>50-2.00(2.11-2.00)</td>
<td>50-1.80(1.86-1.80)</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>228,266(22,644)</td>
<td>187,171(26,404)</td>
<td>82,603(11,499)</td>
<td>96,854(13,940)</td>
<td>133,166(13,147)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>100(100)</td>
<td>99.4(96.8)</td>
<td>98.2(94.6)</td>
<td>99.8(99.2)</td>
<td>100(100)</td>
</tr>
<tr>
<td>(R_{merge})</td>
<td>0.046(0.299)</td>
<td>0.068(0.301)</td>
<td>0.081(0.239)</td>
<td>0.070(0.181)</td>
<td>0.066(0.296)</td>
</tr>
<tr>
<td>Mean (/\sigma(I))</td>
<td>55.2(4.5)</td>
<td>18.8(3.3)</td>
<td>12.9(4.3)</td>
<td>23.1(9.6)</td>
<td>52.1(8.8)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>9.5(6.1)</td>
<td>6.4(3.9)</td>
<td>3.9(3.6)</td>
<td>8.2(7.9)</td>
<td>11.1(11.2)</td>
</tr>
<tr>
<td><strong>MIR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(R_{diff})</td>
<td>0.132</td>
<td>0.161</td>
<td>0.160</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of derivative sites</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Figure of merit</td>
<td></td>
<td></td>
<td>0.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>20-1.50(1.55-1.50)</td>
<td>20-1.60(1.66-1.60)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(R)-factor</td>
<td>0.181(0.224)</td>
<td>0.182(0.244)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(R_{free})</td>
<td>0.191(0.232)</td>
<td>0.194(0.268)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Deviations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond length (Å)</td>
<td>0.005</td>
<td>0.005</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond angle (deg)</td>
<td>1.29</td>
<td>1.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>B factor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avg. main chain (Å(^2))</td>
<td>14.3</td>
<td>13.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avg. side chain (Å(^2))</td>
<td>19.9</td>
<td>18.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avg. waters (Å(^2))</td>
<td>28.9</td>
<td>25.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avg. bestatin (Å(^2))</td>
<td>19.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avg. sulfate (Å(^2))</td>
<td>35.8</td>
<td>46.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EMTS: ethyl mercury thiosalicylate, MeHgCl: methyl mercury chloride, PCMB: \(p\)-chloromercuribenzoate

\(R_{merge} = \sum \sum \frac{|l_{hkl,i} - \langle l_{hkl} \rangle|}{\sum l_{hkl,i}}, \) where \(I_{hkl}\) = observed intensity and \(<I>\) = average intensity for multiple measurements.

Values in parentheses refer to the last resolution shell.

\(R_{diff} = \sum |F_{PH} - |F_P|| / \sum |F_P|, \) where \(|F_{PH}|\) and \(|F_P|\) are derivative and native structure factor amplitudes, respectively.
### Table II Kinetic parameters of the aminopeptidase N

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala-βNA</td>
<td>0.304 ±0.010</td>
<td>63.7 ±2.7</td>
<td>210</td>
</tr>
<tr>
<td>Pro-βNA</td>
<td>0.932 ±0.102</td>
<td>0.743 ±0.043</td>
<td>0.797</td>
</tr>
</tbody>
</table>
Table III Apparent velocity against substrates containing Pro residue.

Apparent velocity against Pro-βNA, Gly-Pro-βNA, and Ala-Phe-Pro-βNA were measured using the aminopeptidase N (APN; 80μg), prolyl aminopeptidase from *B. coagulans* (PAP; 0.01μg), and dipeptidyl aminopeptidase IV from *S. maltophilia* (DPIV; 0.32μg). Assays were carried out using standard assay method described in Materials and Method.

<table>
<thead>
<tr>
<th></th>
<th>Pro-βNA (μmol min⁻¹)</th>
<th>Gly-Pro-βNA (μmol min⁻¹)</th>
<th>Ala-Phe-Pro-βNA (μmol min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) APN</td>
<td>31.2 ±1.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>b) PAP</td>
<td>321 ±28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>c) DPIV</td>
<td>0</td>
<td>165 ±7</td>
<td>0</td>
</tr>
<tr>
<td>d) APN and PAP</td>
<td>369 ±5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>e) APN and DPIV</td>
<td>29.9 ±0.6</td>
<td>82.2 ±0.8</td>
<td>95.4 ±9.2</td>
</tr>
</tbody>
</table>
Fig. 2

(a) (c)

(b) (d)
Fig. 3
Fig. 6

(a)

(b)

(c)
Fig. 7

(a) and (b) show structural diagrams with various residues labeled, such as Asn373, Gln821, Tyr376, Tyr381, Glu320, His297, and His301. The diagrams illustrate the interactions and positions of these residues in the context of the protein structure.

(c) and (d) depict chemical structures with amino acid sequences and bond diagrams, indicating the connectivity and functional groups of the molecules involved.
Fig. 8

(a) Amino Acid Product

(b) Nucleophilic Attack by water

(d) Amine Product

(c)
Aminopeptidase N (proteobacteria alanyl aminopeptidase) from Escherichia coli: Crystal structure and conformational change of the methionine 260 residue involved in substrate recognition

Kiyoshi Ito, Yoshitaka Nakajima, Yuko Onohara, Masahide Takeo, Kanako Nakashima, Futoshi Matsubara, Takashi Ito and Tadashi Yoshimoto

*J. Biol. Chem.* published online August 2, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M605203200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts