Powers, S. G., & Meister, A. (1978a) *J. Biol. Chem. 253*, 1258-1265.
Powers, S. G., & Meister, A. (1978b) *J. Biol. Chem. 253*, 800-803.
Raushel, F. M., & Villafranca, J. J. (1979) *Biochemistry 18*, 3424-3429.
Raushel, F. M., Anderson, P. M., & Villafranca, J. J. (1978) *Biochemistry 17*, 5587-5591.
Raushel, F. M., Rawding, C. J., Anderson, P. M., & Villafranca, J. J. (1979) *Biochemistry 18*, 5562-5566.
Reinhart, G. D. (1983) *Arch. Biochem. Biophys. 224*, 389-401.
Reinhart, G. D., Hartleip, S. B., & Symcox, M. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4032-4036.
Robin, J.-P., Penverne, B., & Herve, G. (1989) *Eur. J. Biochem. 183*, 519-528.
Rubino, S. D., Nyunoya, H., & Lusty, C. J. (1986) *J. Biol. Chem. 261*, 11320-11327.
Trotta, P. P., Burt, M. E., Haschemeyer, R. H., & Meister, A. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2599-2603.
Trotta, P. P., Pinkus, L. M., Haschemeyer, R. H., & Meister, A. (1974) *J. Biol. Chem. 249*, 492-499.
Trotta, P. P., Burt, M. E., Pinkus, L. M., Estis, L. F., Haschemeyer, R. H., & Meister, A. (1978) *Methods Enzymol. 51*, 21-29.
Weber, G. (1972) *Biochemistry 11*, 864-878.
Weber, G. (1975) *Adv. Protein Chem. 29*, 1-83.

Structural Similarity of D-Aminopeptidase to Carboxypeptidase DD and β-Lactamases†

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ABSTRACT: The gene for D-aminopeptidase (*dap*) has been isolated from the bacterium *Ochrobactrum anthropi* SCRC C1-38 [Asano, Y., Nakazawa, A., Kato, Y., & Kondo, K. (1989) *J. Biol. Chem. 264*, 14233-14239] and its nucleotide sequence determined. An expression plasmid pC138DP (4.5 kb) was constructed by placing the gene downstream of the *lac* promoter of pUC19. The amount of the enzyme in the cell-free extract of *Escherichia coli* JM109/pC138DP was elevated to 288 000 units/L of culture, which is about 3600-fold over that of *O. anthropi* SCRC C1-38. The enzyme comprised about 30% of the total extractable cellular protein. The gene consisted of an open reading frame of 1560 nucleotides which specifies a protein of M_r 57 257. The deduced amino acid sequence of the enzyme showed that it is related to carboxypeptidase DD, β-lactamases, and penicillin-binding proteins. Seven mutants of the enzyme were generated by site-specific mutagenesis to explore the roles of the residues of interest, around the sequence Ser61-X_{aa}-X_{aa}-Lys64, where X_{aa} is any amino acid, since the identical sequences also appear in the penicillin-recognizing peptide hydrolases with Ser at the active sites. The mutant enzymes expressed in *E. coli* were purified to homogeneity and kinetically characterized. Replacements of the site at Ser61 and Lys64 yielded mutants showing significantly reduced V_{max} values, while most of the K_m values remained unchanged. Changes at Cys60, which is adjacent to the likely active center Ser61, to Ser and Gly resulted in the production of enzyme less sensitive to PCMB, with almost unaltered V_{max}/K_m values. The enzyme appears to be a serine peptidase rather than a thiol one. The inhibition by PCMB in the wild-type enzyme may have been caused by a formation of a mercaptide bond between Cys 60 and PCMB. Considering that D-aminopeptidase, carboxypeptidase DD (a penicillin-binding protein), and β-lactamase have a common feature in recognizing peptides containing D-amino acid and that the former two catalyze transpeptidation reactions with substrates containing D-alanyl-D-alanine moieties, we propose that the enzyme is a new member of the "penicillin-recognizing enzymes". We showed that the enzyme is actually inhibited by β-lactam compounds, such as 6-APA, 7-ACA, benzylpenicillin, and ampicillin, although they are not the substrate for the enzyme. The relationship between the primary structures and the reactions catalyzed by D-aminopeptidase and other serine hydrolases β-lactamases and carboxypeptidase DD is discussed. To our knowledge, this enzyme is the first example of an aminopeptidase with Ser at the active site.

We have been exploiting new enzymes to use as catalysts in organic synthesis (Asano et al., 1982, 1987, 1989a-d, 1990, 1991). We have previously reported on the purification, characterization, and properties of novel D-stereospecific aminopeptidase and an D-stereospecific amino acid amidase from bacterial isolates from soil, *Ochrobactrum anthropi* SCRC C1-38 (Asano et al., 1989a,b) and *O. anthropi* SCRC SV3 (Asano et al., 1989c), respectively. The former enzyme is specific toward peptides with a free D-amino acid at the NH_2 terminus and D-amino acid amides, etc., and thus was named as "D-aminopeptidase" (Asano et al., 1989b), while the latter was specific toward D-amino acid amides with bulkier substituents (Asano et al., 1989c). The enzymes can be used in

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an optical resolution of amino acid amides to yield D-amino acids (Asano et al., 1989a), and the D-aminopeptidase catalyzes the syntheses of D-amino acid *N*-alkylamide (Kato et al., 1989) and D-alanine oligopeptides (Kato et al., 1990) in water and in organic solvents.

The enzyme appeared to be unique because of its lower substrate requirement and its less frequent occurrence as a bacterial thiol peptidase compared to those of the metal-containing ones (Asano et al., 1991; Brenner, 1988; DeLange et al., 1971). We cloned the gene for the enzyme in *Escherichia coli*, not only to overproduce the enzyme, but also to study the structural relationship to other proteins. We have now deduced a complete amino acid sequence of D-aminopeptidase by gene sequencing. The sequence of the enzyme showed striking similarities with the *Streptomyces* R61 extracellular carboxypeptidase DD (EC 3.4.16.-) (Duez et al., 1987; Ambler, 1980) and β-lactamases (EC 3.5.2.6) (Frère & Joris, 1985; Lindberg & Normark, 1986), which are involved in the synthesis of bacterial peptidoglycan and the hydrolysis of its analogue, β-lactam compounds, respectively.

We cloned and sequenced the gene for the D-aminopeptidase from *O. anthropi* SCRC C1-38, to determine the similarity of its primary structure with that of other proteins, introduced amino acid substitution at specific sites sharing similarities with the catalytic site of the "penicillin-recognizing enzymes" (Joris et al., 1986; Frère et al., 1988), performed kinetic characterization of the mutants, and studied the inhibition by β-lactam compounds. We present evidence that D-aminopeptidase is a new member of this class of enzymes.

Materials and Methods

Materials. DNA polymerase (*E. coli*), pUC18, pUC19, pUC118, pUC119, M13 mp18, M13 mp19, and kits for M13 sequencing, kilo-sequencing, 7-deaza sequencing, DNA blunting, and site-specific mutagenesis (Mutan-K) were purchased from Takara Shuzo (Kyoto, Japan). Restriction endonucleases, T4 polynucleotide kinase (T4-infected *E. coli*), and dNTPs were purchased from Takara Shuzo and Toyobo (Osaka, Japan), and calf intestine alkaline phosphatase was from Boehringer Mannheim (Germany). [γ-^{32}P]dCTP was from Amersham. Butyl-Toyopearl and HPLC columns, DEAE-5PW and phenyl-5PW, were purchased from Tosoh Corp. (Tokyo, Japan), and marker DNA was from Nippon Gene (Toyama, Japan). IPTG,[1] ribonuclease A (bovine pancreas), proteinase (*Streptomyces griseus*), *E. coli* DNA, (D-Ala)$_2$, (D-Ala)$_3$, N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine, ampicillin, benzylpenicillin, 6-APA, 7-ACA, and D-amino acid oxidase (EC 1.4.3.3, hog kidney) were purchased from Sigma, and calf thymus DNA was from Boehringer Mannheim. D-Alanine-*p*-nitroanilide and D-alanine amide hydrochloride were purchased from Bachem (Bubendorf, Switzerland). *tert*-Butyl dicarbonate was purchased from Kokusan Chemicals (Japan) and *N*-(nitrosomethyl)urea from ICN K&K Laboratories, Inc. (United Kingdom). All other chemicals were of analytical grade.

Substrate Synthesis. ^{1}H NMR spectra were measured by a Varian EM-390 using tetramethylsilane or 3-(trimethylsilyl)propanesulfonic acid sodium salt as a reference.

(*A*) *Benzylpenicillin Methyl Ester.* Benzylpenicillin potassium salt (5.19 mmol) was converted to free form with 10% phosphoric acid and extracted with Et_2O. To this was added an excess amount of ethereal diazomethane at room temperature. Crude product was purified by column chromatography on silica gel (hexane/ethyl acetate = 85/15 and next Et_2O) to give a pale yellowish powder in a yield of 77.5%: ^{1}H NMR ($CDCl_3$) δ 1.43 (s, 3 H), 1.45 (s, 3 H), 3.25 (s, 2 H), 3.70 (s, 3 H), 4.34 (s, 1 H), 5.48 (m, 1 H), 5.63 (m, 1 H), 6.40 (d, 1 H), 7.30 ppm (m, 5 H); MS (*m*/*z*) 28 (10.2), 91 (85.8), 160 (11.2), 174 (100), 234 (2.5), 289 (0.5), 348 (M^+, 2.1).

(*B*) *Boc-ampicillin.* To a stirred solution of ampicillin sodium salt (10 mmol) in 66% aqueous dioxane (100 mL) was added a dioxane solution (50 mL) of di-*tert*-butyl dicarbonate (15 mmol) at 0 °C. Stirred overnight at room temperature, the mixture was evaporated and distributed to 5% $NaHCO_3$ and Et_2O. An aqueous phase was washed with Et_2O and adjusted to pH 2 with phosphoric acid at 0 °C. The precipitate formed was extracted with ethyl acetate, and *N*-Boc-ampicillin was obtained as a pale yellowish powder followed by a recrystallization with ethyl acetate and petroleum ether in a yield of 78%: ^{1}H NMR ($CDCl_3$) δ 1.42 (s, 9 H), 1.50 (s, 3 H), 1.53 (s, 3 H), 4.30 (s, 1 H), 5.30 (m, 1 H), 5.48 (m, 1 H), 5.59 (m, 1 H), 6.00 (br, 1 H), 7.00 (d, 1 H), 7.66 (m, 5 H), 10.0 ppm (br, 1 H); MS (*m*/*z*) 56 (87.2), 100 (59.5), 150 (63.7), 206 (30.3), 234 (34.9), 347 (2.6), 449 (M^+, 0.3).

(*C*) *Boc-ampicillin Methyl Ester.* Boc-ampicillin (2.22 mmol) was esterified with diazomethane as described above and recrystallized with Et_2O and petroleum ether gave methyl ester as a pale yellowish powder in a yield of 87.6%: ^{1}H NMR ($CDCl_3$) δ 1.43 (s, 9 H), 1.50 (s, 3 H), 1.52 (s, 3 H), 3.73 (s, 3 H), 4.42 (s, 1 H), 5.25 (d, 1 H), 5.50 (m, 1 H), 5.66 (m, 2 H), 6.83 (d, 1 H), 7.33 ppm (m, 5 H); MS (*m*/*z*) 57 (85.4), 106 (73.3), 150 (24.0), 174 (100), 234 (2.4), 407 (0.7), 463 (M^+, 0.5).

(*D*) *Ampicillin Methyl Ester.* Boc-ampicillin methyl ester (1.62 mmol) was deprotected with trifluoroacetic acid (50 mL) at room temperature for 1 h. Azeotropic evaporation with toluene and methanol of the reaction mixture gave a brown powder in a yield of 90.6%: ^{1}H NMR (CD_3OD) δ 1.50 (s, 3 H), 1.53 (s, 3 H), 3.73 (s, 3 H), 4.70 (m, 2 H), 5.18 (m, 1 H), 5.70 (m, 1 H), 7.53 ppm (m, 5 H); MS (*m*/*z*) 28 (52.0), 106 (100), 174 (85.7), 270 (22.1), 290 (0.52), 346 (0.96), 363 (M^+, 0.23).

(*E*) *Boc-6-aminopenicillanic Acid.* 6-APA (20 mmol) was *N-tert*-butoxycarbonylated by the above manner. Boc-6-APA was obtained as a colorless powder followed by a recrystallization with Et_2O and petroleum ether in a yield of 94.9%: ^{1}H NMR ($CDCl_3$) δ 1.48 (s, 9 H), 1.63 (s, 3 H), 1.70 (s, 3 H), 4.36 (s, 1 H), 5.50 (m, 2 H), 7.60 (d, 1 H), 10.8 ppm (br, 1 H); MS (*m*/*z*) 56 (100), 91 (100), 92 (100), 160 (14.6), 216 (1.5), 260 (1.2), 316 (M^+, 0.5).

(*F*) *Boc-6-aminopenicillanic Acid Methyl Ester.* Boc-6-APA (10 mmol) was esterified with diazomethane as described above. Boc-6-APA-OCH_3 was obtained as a colorless powder followed by a recrystallization with ethyl acetate/Et_2O/petroleum ether in a yield of 65%: ^{1}H NMR ($CDCl_3$) δ 1.45 (s, 9 H), 1.51 (s, 3 H), 1.63 (s, 3 H), 3.75 (s, 3 H), 4.40 (s, 1 H), 5.10–5.50 (m, 1 H), 5.60 ppm (d, 1 H); MS (*m*/*z*) 57 (84.1), 75 (21.2), 119 (10.1), 174 (100), 274 (4.6), 330 (M^+, 0.2).

(*G*) *6-Aminopenicillanic Acid Methyl Ester.* Boc-6-APA-OCH_3 (3 mmol) was deprotected with trifluoroacetic acid and purified as described above, and the deprotected methyl ester

[1] Abbreviations: bp, base pair(s); kb, kilobase pair(s); 6-APA, 6-aminopenicillanic acid; 7-ACA, 7-aminocephalosporanic acid; -OCH_3, methyl ester; Boc, *tert*-butoxycarbonyl; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; IPTG, isopropyl 1-thio-β-D-galactopyranoside; DNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; PCMB, *p*-(chloromercuri)benzoate; PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropyl phosphorofluoridate; *bla*, gene coding for β-lactamase; *dap*, gene coding for D-aminopeptidase; *lac* P/O, *lac* promoter–operator; PBP, penicillin-binding proteins.

was obtained as a pale yellowish powder followed by a recrystallization with Et_2O and methanol in a yield of 96.3%: ^1H NMR (CD_3OD) δ 1.48 (s, 3 H), 1.53 (s, 3 H), 3.80 (s, 3 H), 4.37 (d, 1 H), 4.63 (s, 1 H), 4.93 (br), 5.73 ppm (d, 1 H); MS (m/z) 28 (77.1), 114 (68.2), 124 (100), 174 (54.4), 215 (18.6), 230 (M^+, 16.4).

Bacterial Strains, Plasmids, and Cultivation. *O. anthropi* SCRC C1-38,[2] isolated from soil with a medium containing D-alanine amide as a sole source of nitrogen (Asano et al., 1989a), was kept in the stock culture (No. 0115) of the Sagami Chemical Research Center, 4-4-1 Nishi-Ohnuma, Sagamihara, Kanagawa, 229 Japan. The strain was cultivated with rotary shaking in a medium containing 5 g of yeast extract (Oriental Yeast, Tokyo, Japan), 5 g of peptone (Kyokuto, Tokyo, Japan), 1 g of glucose, and 1 g of K_2HPO_4 per 1 L of tap water, pH 7.0, for 10 h at 30 °C. *E. coli* JM 109, JM 103 (Yanisch-Perron et al., 1985), MV1184 (Vieira & Messing, 1987), and BW313 (*dat*$^-$ *ung*$^-$) (Kunkel, 1985) were used as hosts and cultivated with shaking in LB medium (Maniatis et al., 1982), supplemented with 50 μg/mL ampicillin, for 12 h at 37 °C. pUC18, pUC19 (Vieira & Messing, 1982), pUC118, and pUC119 (Yanisch-Perron, 1985) were used as vectors. For the maximum expression of D-aminopeptidase by *E. coli* JM109/pC138DP, the transformant was cultivated in the LB medium, supplemented with 10 g of glycerol and 2 mg of thiamine hydrochloride per liter.

DNA Techniques. For the isolation of DNA, cells of *O. anthropi* SCRC C1-38 were harvested ater 10-h cultivation at an optical density at 610 nm of 0.8 unit. Total DNA of *O. anthropi* was isolated following the procedure described by Doi (Rodriguez & Trait, 1983) after a modification, including spheroplast formation by lysozyme, cell breakage by sonication and SDS treatment, phenol extraction, ribonuclease A and proteinase treatments, and ethanol precipitation. For sonication, cells were suspended in 0.1 M potassium phosphate, pH 7.0, and disrupted for 1 min in a Kubota-Syoji 9-kHz sonic oscillator. Plasmids were isolated by the alkaline extraction procedure (Birnboim & Doly, 1979). Restriction endonucleases and other enzymes were used as recommended by the suppliers. Other techniques used were essentially as described by Maniatis et al. (1982), Rodriguez and Tait (1983), and Davis et al. (1986).

Cloning and Screening of Recombinants. The gene for D-aminopeptidase from *O. anthropi* was isolated from a plasmid pool containing *Eco*RI fragments of *O. anthropi* DNA ligated into the dephosphorylated *Eco*RI site of pUC19 (Asano et al., 1991). Competent *E. coli* JM109 cells were prepared and transformed with the ligation mixture according to the method of Hanahan (1983), and transformants were selected for expression of ampicillin resistance.

The visualization of the D-aminopeptidase activity expressed in the transformants was carried out by monitoring the formation of the yellow color of *p*-nitroaniline from D-alanine-*p*-nitroanilide (Asano et al., 1989b), by a method described previously (Asano et al., 1987b) after a modification. A sheet of nitrocellulose filter (Advantec, 82 mm in diameter) with 500–1000 colonies of recombinants was floated, after lysozyme treatment and repetitive freeze–thawing, on the surface of 1 mL of 10 mM D-alanine-*p*-nitroanilide solution in water and was let stand at room temperature. Colonies that developed the yellow color of *p*-nitroaniline in a few minutes were picked up. The D-aminopeptidase activity in the cell-free extract of the positive transformants was measured under the standard assay conditions. D-Aminopeptidase-positive *E. coli* transformants were also visualized by pouring the 10 mM D-analine-*p*-nitroanilide solution directly onto the colonies grown on the LB plates. The positive transformants were quickly stained yellow. The host *E. coli* cells did not show the activity.

Subcloning and Construction of an Expression Plasmid pC138DP. About 2 of the approximately 10000 transformants exhibited D-aminopeptidase activity as shown by the development of the yellow color on the nitrocellulose filter screening assay. Rapid isolation of plasmids from the 2 transformants revealed that both contained about a 10-kb insert. The insert DNA fragment from one of the plasmids designated pC138E, was isolated by agarose electrophoresis and partially digested with *Hin*dIII. The mixture of digested DNA was subcloned with *Hin*dIII-digested and dephosphorylated pUC19 and introduced to *E. coli* by transformation. A D-aminopeptidase-positive clone was isolated and found to harbor a plasmid designated pC138H with about a 2.4-kb DNA insert. The pC138H could transform *E. coli* into D-aminopeptidase positive. pC138H was digested with *Sac*I and *Sal*I and then subjected to exonuclease III digestion (Heinkoff, 1984) to shorten the insert DNA from the 5′ end using a kit (Kilo-Sequencing Kit, Takara Shuzo, Japan) to give pC138K, which contained a 2.2-kb insert at the *Eco*RI–*Pst*I site of pUC19.

By nucleotide sequencing of the *dap* gene, a *Dra*I site was identified 50 bp upstream of the initiation codon ATG. A 1.84-kb *Dra*I–*Pst*I fragment containing the *dap* gene without the endogenous promoter was isolated by removing about a 210 bp DNA fragment upstream of the *Dra*I site, and the DNA ends were made blunt by T4 DNA polymerase [DNA blunting kit (Takara Shuzo, Japan)]. The DNA fragment was ligated to the dephosphorylated *Sma*I site of pUC19 and used to transform *E. coli* JM109. The transformants were screened by ampicillin resistance and the expression of the D-aminopeptidase activity. A plasmid designated pC138DP (4.5 kb) was isolated from a D-aminopeptidase-positive *E. coli* clone. Nucleotide sequencing revealed that the *dap* gene is located downstream of the *lac* promoter of pUC19.

Nucleotide Sequencing Analysis. The fragment to be sequenced was subcloned to either M13 mp18, M13 mp19, pUC118, or pUC119 by standard procedures. To generate shorter clones suitable for sequencing, the exonuclease III deletion method by the Kilo-Sequencing Kit (Takara Shuzo, Japan) was used. The sequence was determined by the dideoxy chain-termination procedure of Sanger (Sanger et al., 1977), using kits for M13 sequencing and 7-deaza sequencing.

The 5′-terminal region of the coding sequence of the *dap* gene was localized by using a ^{32}P-end-labeled 24 mixture of 14-mer probe [5′-d[TT(T/C)AA(A/G)CT(A/G)TG(A/G/T/C)AG]-3′], which was chemically synthesized by the phosphoramidite method using an Applied Biosystems Model 391 DNA synthesizer and then labeled with T4 polynucleotide kinase and [α-^{32}P]ATP. This probe was synthesized according to the amino acid sequence, Lys2-Phe-Asp-Thr-Ser6, which was found in the N-terminal amino acid sequence of the purified *O. anthropi* D-aminopeptidase (Asano et al., 1989b). Southern blot analysis of restriction fragments of pC138H showed that the ^{32}P-end-labeled probe mixture hybridized with the insert DNA.

Site-Specific Mutagenesis. Site-specific mutagenesis was carried out using mutagenic oligonucleotides, synthesized as described above. The method of Kunkel (Kunkel, 1985; Kunkel et al., 1987) using gapped heterodoplex by the Mutan

[2] *Ochrobactrum anthropi* [formerly *Achromobacter* sp (Asano et al., 1989a) or CDC-Vd (Holmes et al., 1988)] SCRC C1-38 is available from American Type Culture Collection (12301 Parklawn Dr., Rockville, MD 20852) with Accession Number ATCC 49237.

K Kit (Takara shuzo, Japan) has been employed. The DNA fragment containing a *dap* gene generated by digestion of pC138K with *Eco*RI and *Pst*I was filled in with DNA polymerase and subcloned to the dephosphorylated *Sma*I site of pUC119, and a plasmid with the opposite orientation against the *lac* promoter was selected. The plasmid was used to transform *E. coli* MV1184, in order to produce a single-strand DNA which is for the most part complementary with mismatching to the designed mutagenic oligonucleotides. The sequences (5′→3′) of synthetic oligonucleotides are as follows: 1, d(GCCGATCTCTTCGGTCAG) (location on the *dap* gene is from 174 to 191 in Figure 1; Cys60 → Ser60); 2, d(TGCCGATCGGTTCGGTCA) (173–190; Cys60 → Gly60); 3, d(CGATCTGTTGTGTCAGCAAGC) (176–196; Ser61 → Cys61); 4, d(CCGATCTGTGGGGTCAGCAAG) (175–195; Ser61 → Gly61); 5, d(GATACGCGGTTTCCGATCTGT) (163–183; Met57 → Phe57); 6, d(GTCAGCAACCAGTTTACC) (187–104; Lys64 → Asn64); and 7, d(GTTTACCTCTGCTGTGCT) (198–215; Cys68 → Ser68). *E. coli* BW313 (*dut*⁻ *ung*⁻) was transformed by the plasmid to yield a uracil-enriched template of single-stranded DNA. The mismatching synthetic oligonucleotides were phosphorylated at the 5′-terminus by T4 polynucleotide kinase and annealed with the single-stranded DNA. After DNA polymerization with T4 DNA polymerase, the double-stranded DNA synthesized was used to transform *E. coli* BMH-71-18 *mut* S (*ung*⁺). Single-stranded DNA from the *E. coli* mutant was used to transform *E. coli* MV1184 to give a mature double-stranded mutant DNA. All mutations were confirmed by sequencing the sites with a synthetic primer 5′-d(TGCAACACGCCTGGGG)-3′, the location on the *dap* gene of which is 111–125 in Figure 1.

Purification of the Mutant Enzymes and N-Terminal Amino Acid Sequencing. DNA fragments of the mutated plasmids were isolated by electroelution and ligated to the *Eco*RI and *Pst*I sites of pUC18. Transformants harboring the natural and mutant plasmids were cultivated in 100 mL of LB medium, containing 50 μg/mL ampicillin. The cells were harvested and suspended in 5 mL of 0.01 M potassium phosphate buffer, pH 7.0. The cells were disrupted for 15 min with a 20-kHz ultrasonic oscillator, UCD-130 (Tosho Denki, Tokyo, Japan). The disrupted cells were centrifuged at 1500*g* for 20 min. All enzyme purifications were carried out at temperatures below 4 °C, involving ammonium sulfate fractionation (30–90%), column chromatographies on butyl-Toyopearl (volume 50 mL, eluted with a gradient of ammonium sulfate from 30% to 15%), and HPLC on DEAE-5PW, eluted as described previously (Asano et al., 1989b). Mutant D-aminopeptidases 1, 2, 4, and 5 were thus isolated. Because the expression was low for mutant enzymes 3, 6, and 7 with the plasmids constructed, they were digested with *Dra*I and *Pst*I and the fragments containing the *dap* gene were isolated. The DNA ends were made blunt by DNA polymerase and ligated to *Sma*I-digested and dephosphorylated pUC19, as described above for the construction of the expression plasmid. Expressed enzymes were purified to homogeneity as described above for the *E. coli* transformants. Mutant enzyme 4 was purified by monitoring a band corresponding to the active enzyme, using SDS–polyacrylamide gel electrophoresis. A vertical slab gel apparatus (DPE 2210, Daiichi Kagaku Yakuhin, Tokyo, Japan) was used for polyacrylamide gel electrophoresis in the presence and absence of SDS. Gel electrophoresis was carried out as described by Laemmli (1970). The gels used were prepared by Daiichi Kagaku Yakuhin; acrylamide concentration was 4% in the stacking gel and 10% in the main gel part. The size of the gel was 9.0 cm × 8.4 cm in height and width and 1.0 mm in thickness. Coomassie Brilliant Blue (0.25%) in methanol/acetic acid/water (5:1:5) was used to visualize the proteins. Destaining was carried out with 7% acetic acid.

To determine the NH_2-terminal amino acid sequence, the enzyme sample was passed through a TSK phenyl-5PW column (0.75 × 7.5 cm, Tosoh Corp., Tokyo, Japan) and fractionated with a linear gradient of 20–80% (v/v) acetonitrile containing 0.05% (v/v) trifluoroacetic acid. The enzyme sample (about 300 μg) was analyzed with automatic protein sequencer 470A (Applied Biosystems, Foster City, CA) (Hunkapiller et al., 1983).

Enzyme Assay. The enzyme activity was assayed by the hydrolysis of D-alanine amide (Asano et al., 1989b) or by the formation of *p*-nitroaniline from D-alanine-*p*-nitroanilide at 405 nm as follows. A reaction mixture (1.0 mL) containing 5 mM D-alanine-*p*-nitroanilide, 100 mM Tris-HCl, pH 8.0, and the enzyme was monitored by the change in absorbance at 405 nm with a Hitachi 228A spectrophotometer. A linear change in absorbance for at least 1 min was employed in the kinetic study. Typically, the amount of the enzyme (as a homogeneous enzyme of the native structure) in the reaction mixture was 0.004–0.2 μg, causing a change of 0.01–0.5 absorbance unit/min. When the specific activities of mutant enzymes were measured, the amount of the enzymes was increased so as to give the same absorbance change as the natural one. One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol of D-alanine from D-alanine amide per minute. This was calculated from the relative reactivity toward D-alanine-*p*-nitroanilide as compared with that toward D-alanine amide (=96:100) (Asano et al., 1989b). The K_m and V_{max} values were calculated from Lineweaver–Burk plots with computer-aided fitting. Protein was determined by the method of Lowry (Lowry et al., 1951), by Coomassie Brilliant Blue staining (Bio-Rad), or from the absorbance at 280 nm.

β-Lactamase activity was assayed as follows. A solution (1.0 mL) containing 10 units of D-aminopeptidase, 50 mM of buffer of several pHs, and 5 mM of β-lactam compounds (ampicillin, benzylpenicillin, 6-APA, 7-ACA, etc.) was incubated at 30 °C for various incubation times. The reaction mixture was spotted on silica gel thin-layer chromatography (TLC) and developed with a solvent system (1-butanol/acetic acid/ethanol/water = 80/15/40/24 v/v). The substrates and degradation products were detected by UV and/or spraying ninhydrin. No more than 0.1–1% of the degradation products relative to the substrates could be detected by this method. Carboxypeptidase DD activity was examined by the method of Ghuysen (Frère et al., 1976). A reaction mixture (1.0 mL) containing 10 units of the enzyme, 5 mM N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine, and 50 mM buffer of various pHs was incubated at 30 °C for several hours. Transpeptidation activity was assayed using N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala as an acyl donor and glycylglycine as an acceptor (Frère et al., 1976). A reaction mixture (1.0 mL) containing 10 units of the enzyme, 2 mM N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala, and 20 mM glycylglycine in 50 mM buffer of various pHs was incubated at 30 °C for several hours. D-Alanine formed by transpeptidation reaction from the substrate was analyzed with a system containing D-amino acid oxidase, peroxidase, 4-aminoantipyrine, and phenol (Asano et al., 1989b).

Inhibition of the Enzyme by β-Lactam Compounds. A reaction mixture (1.0 mL) containing 0.1–0.15 unit of the purified wild-type D-aminopeptidase, 50 mM Tris-HCl, pH

8.0, and inhibitor of various concentrations was incubated at 30 °C for 30 min. The reaction was started by addition of the substrate at various concentrations (0–10 mM) and incubated at 30 °C. When D-alanine-*p*-nitroanilide was used as substrate, the absorbance change at 405 nm was followed with a double-beam spectrophotometer as described above. When D-alanine oligomers were used as substrates, an aliquot of the reaction mixture was assayed for the formation of D-alanine in an assay system utilizing the D-amino acid oxidase system (Asano et al., 1989b), after termination by boiling. Inhibition type was determined from Lineweaver–Burk plots with several concentrations of substrates and inhibitors. The K_i values were estimated by replotting [I] versus 1/[V] or 1/[S].

Results

Cloning and Nucleotide Sequencing of the dap Gene. The gene for D-aminopeptidase from *O. anthropi* was isolated from a plasmid pool containing *Eco*RI fragments of *O. anthropi* chromosomal DNA ligated into the *Eco*RI site of pUC19 and introduced in *E. coli* JM109 by transformation. Transformants expressing ampicillin resistance and the D-aminopeptidase activity were screened by the development of yellow color from D-alanine-*p*-nitroanilide. The plasmids pC138E, pC138H, and pC138K, containing about 10-, 2.4-, and 2.2-kb inserts, respectively, can transform *E. coli* into D-aminopeptidase positive. The enzyme activities of *E. coli* transformants were fully detected, even when the inserts of the three plasmids were ligated to a vector pUC18, making the direction of the transcription from the *lac* promoter opposite to that of the original plasmid. This shows that the transcription in the plasmids is governed by the endogenous promoter originating from *O. anthropi*.

Various mutants of the plasmid deleted from the 5′-terminus of the *Eco*RI site of the 2.2-kb insert of pC138K were generated by using the kilo-sequencing kit. To locate which DNA fragment is encoding for the N-terminus of D-aminopeptidase, a synthetic mixed probe, corresponding to the N-terminal amino acid sequence (Asano et al., 1989b), was labeled with [γ-^{32}P]dCTP by T4 polynucleotide kinase and hybridized with the deletion mutants. The full 2.2-kb insert was sequenced in both strands. The open reading frame of the *dap* gene has the initiation codon (ATG) and the termination codon (TGA), encoding an open reading frame of 1560 nucleotides which specifies a protein of M_r 57 257 as shown in Figure 1. Ahead of the initiation codon, there is a sequence GAG, similar to the ribosomal binding site (Shine–Dalgarno sequence) (Shine & Dalgarno, 1974; Storomo et al., 1982). The 25 amino acid residues (underlined in Figure 1) coincided with those identified by the N-terminal amino acid sequencing of the purified enzyme (Asano et al., 1989b). The molecular weight of the encoded protein monomer of D-aminopeptidase estimated from the deduced amino acid sequence was in agreement with the molecular weight of 59 000 determined by SDS–polyacrylamide gel electrophoresis of the purified enzyme (Asano et al., 1989b). The enzyme produced by the *E. coli* transformant was purified to homogeneity, and the N-terminal amino acid sequence was determined. The amino acid sequence was the same as that of the native enzyme as described in the next section. We concluded that this open reading frame coded for the *O. anthropi* D-aminopeptidase. The sequenced DNA fragment (2051 bp) contains 54.4% G+C, which is similar to the G+C value of *O. anthropi* DNA (56–59 mol %) (Holmes et al., 1988). The codon usage of the *dap* gene is not biased.

Expression of D-Aminopeptidase in E. coli. By nucleotide sequencing, a *Dra*I site was identified 50 bp upstream of the initiation codon ATG. A 1.84-kb fragment containing the *dap* gene was isolated and ligated to the *Sma*I site of pUC19 to give pC138DP (4.5 kb), in which the *dap* gene is located downstream of the *lac* promoter of pUC19. The direction of the open reading frame was confirmed by gene sequencing to coincide with that of the *lac* promoter. Figure 2 shows the restriction map of pC138DP.

Overproduction of D-aminopeptidase was observed with *E. coli* transformant harboring the expression plasmid pC138DP in LB medium supplemented with 1% glycerol and a trace amount of thiamin, without ampicillin and IPTG. The amount of the enzyme in the cell-free extract of *E. coli* JM109/pC138DP was elevated to 288 000 units/L of culture, which is about 3600-fold over that of *O. anthropi* SCRC C1-38 (80 units/L) (Asano et al., 1989b). The enzyme appears to be soluble and in an active form in the cells, because the whole cells of *E. coli* transformant gave the same enzyme activity as the cell-free extract. The enzyme comprised about 30% of the total extractable cellular protein in a soluble form. When the cell-free extract of the *E. coli* transformant was subjected to polyacrylamide gel electrophoresis, two bands with the enzyme activity were detected. One of them was electrophoresed at the same M_r of the subunit in a SDS–polyacrylamide gel. One of the bands in the polyacrylamide gel in the absence of SDS corresponded with the native enzyme from wild-type *O. anthropi*. Another band which appeared toward the cathode side of the native enzyme was separated by butyl-Toyopearl column chromatography and further purified to homogeneity by HPLC with DEAE-5PW column. The N-terminal amino acid sequence thus revealed had 7 amino acids more than the native one starting from Val, i.e., Val-X_{aa}-Pro-Glu-X_{aa}-Ile-Met-Ser-Lys-Phe-Asp-Tyr-X_{aa}-Ser. The fused enzyme was purified to homogeneity, and the specific activity (542 units/mg) did not differ greatly from that of the enzyme from wild-type *O. anthropi* (590 units/mg) (Asano et al., 1989b). The reason for the existence of the fused enzyme remains unknown. Transcription of the *dap* gene on pC138DP was clearly dependent on the *lac* promoter of pUC19, since D-aminopeptidase activity was not expressed when the fragment containing the gene was ligated to the same site of pUC18. Addition of IPTG was rather inhibitory. The high expression of the enzyme without IPTG would probably be supported by the stability of its mRNA and the gene product.

Deduced Primary Sequence and Similarities with Other Proteins. Computer alignments by the method of Korn et al. (1977) showed that the deduced primary structure of D-aminopeptidase is considerably similar to the two highest scoring sequences of those of *Streptomyces* R61 carboxypeptidase DD (Duez et al., 1987) and class C β-lactamases (Jaurin & Grundström, 1981; Lindberg & Normark, 1986; Galleni et al., 1988). Figure 3 shows the results of the alignment of the primary structure of D-aminopeptidase with those of *Streptomyces* R61 carboxypeptidase DD (Duez et al., 1987) and class C β-lactamase from *E. coli* K12 (Lindberg & Normark, 1986). Considerable similarity was seen between these enzymes, although not very high. Between D-aminopeptidase and carboxypeptidase DD, 27% of the residues in the same area (287 amino acids) are identical. There is 22% similarity between D-aminopeptidase and class C β-lactamase from *E. coli* (Lindberg & Normark, 1986). Further survey of the literature on this class of enzymes showed that the enzyme was structurally related to the penicillin-recognizing enzymes, including carboxypeptidase DD (Duez et al., 1987) class C β-lactamases (Jaurin & Grundström, 1981; Knott-

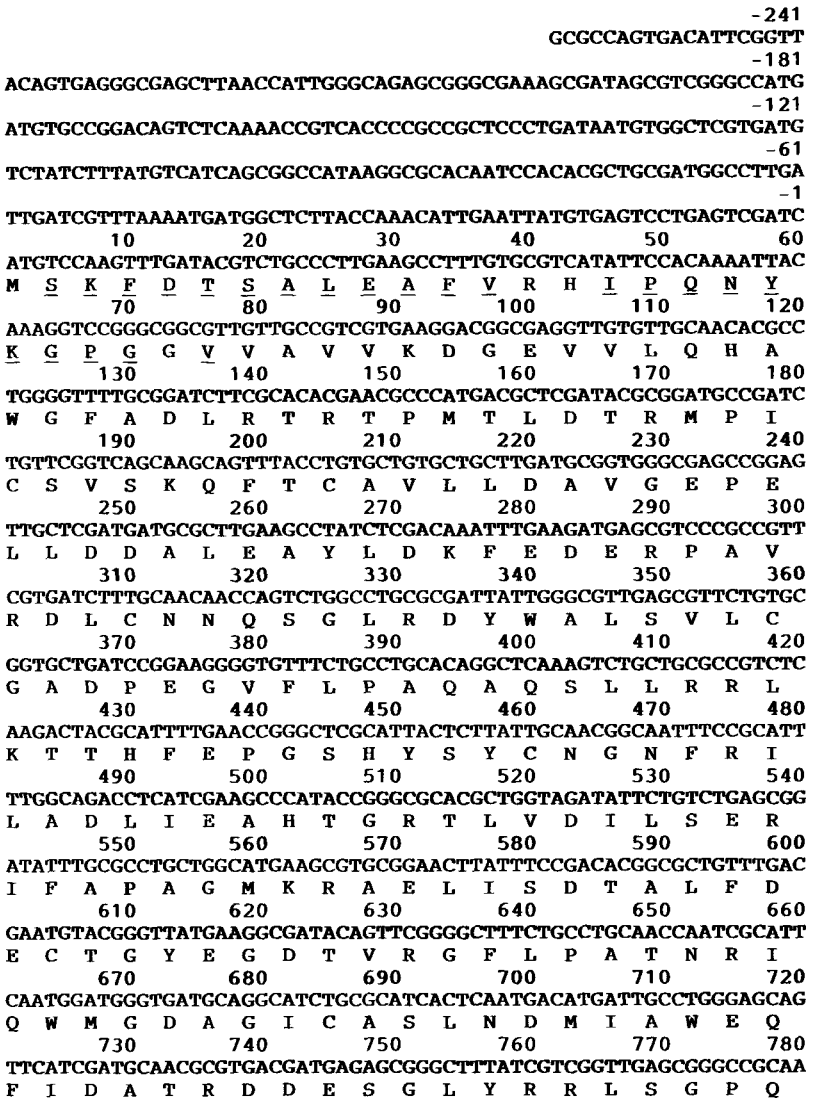

FIGURE 1: Nucleotide sequence of the *dap* gene. An open reading frame of 1560 bp (520 amino acids) shown with the deduced amino acid sequence. The first letter of the putative translation initiation codon is designated as +1. The arrow and asterisk indicate the start and stop codons, respectively. Underlined are those amino acid residues which are found in the N-terminal sequence determined with purified D-aminopeptidase.

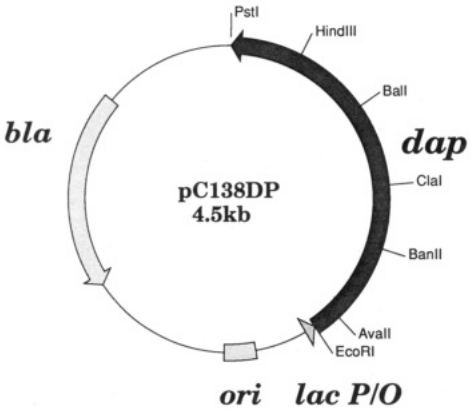

FIGURE 2: Restriction map of pC138DP.

Hunziker et al., 1982; Joris et al., 1985; Lindberg & Normark, 1986; Galleni et al., 1988), class A β-lactamases (Ambler & Scott, 1978; Neuberger, 1981; Sloma & Gross, 1983; Chan, 1986; Joris et al., 1987; Lenzini et al., 1987; Dehottay et al., 1987), penicillin-binding proteins (Yocum et al., 1980; Nakamura et al., 1983; Broome-Smith et al., 1985; Asoh et al., 1986; Song et al., 1987; Broome-Smith et al., 1988; Spratt, 1988), and class D β-lactamases (Dale et al., 1985, Huovinen et al., 1988), as shown in Figure 4. The sequence Ser-X_{aa}-X_{aa}-Lys is perfectly conserved among this class of enzymes. With carboxypeptidase DD and class C β-lactamase, the sequences at the highly similar areas, Ser-Val-X_{aa}-Lys-X_{aa}-Phe-X_{aa}-Ala-X_{aa}-Val-Leu-Leu (Duez et al., 1987) and Ser-Val-Ser-Lys-X_{aa}-Phe-Tyr (Lindberg & Normark, 1986), respectively, are well conserved. Furthermore, some of the residues scattered around the conserved region are similar among the enzymes. The Met57 located four residues upstream from the Ser61 is found similarly in the carboxypeptidase DD and β-lactamases as a hydrophobic residue, Phe. A common observation between D-aminopeptidase and the β-lactamase is that the consensus sequence is located around 60 from the N-terminus of the enzymes (Joris et al., 1986). A triad sequence His167-Tyr-Gly of D-aminopeptidase also appears to be conserved among this class of enzymes (Joris et al., 1986).

Site-Specific Mutagenesis of D-*Aminopeptidase.* The computer alignment study of the primary structure of D-aminopeptidase, which showed rather low structural similarities with penicillin-recognizing enzymes, gave rise to two questions. One is whether they happened to share structural similarities by chance or are both structurally and functionally related. The other is on the role of the highly conserved sequence around Ser-X_{aa}-X_{aa}-Lys: We previously concluded that the enzyme would probably be classified as a thiol peptidase (Asano et al., 1989b). This observation is in contradiction with the result of the alignment study, since all members of this class of enzymes have been proved to have a Ser residue at their active center (Joris et al., 1986; Frère et al., 1988).

Seven mutant enzymes were generated by site-specific mutagenesis. The conserved amino acids around the likely active sites of the enzyme were changed. The site at Cys60 was changed to Ser (mutant 1) and Gly (mutant 2), Ser61 to Cys (mutant 3) and Gly (mutant 4), Met57 to Phe (mutant 5), Lys64 to Gln (mutant 6), and Cys68 to Ser (mutant 7). The mutant enzymes expressed in *E. coli* were all purified to homogeneity as shown in Figure 5. For mutants 3, 6, and 7, two active forms of the enzyme were detected by polyacrylamide gel electrophoresis as described in Materials and Methods. The enzymes eluted at the same position with the native enzyme in the polyacrylamide gel, excluding the fused enzyme described above, were purified to homogeneity and used for activity measurement.

Table I: Specific Activities of Natural and Mutant D-Aminopeptidases Generated by Site-Specific Mutagenesis

mutant	sequence	V_{max} (units/mg)	K_m (mM)	V_{max}/K_m [units/(mM·mg)]
Natural	LDTRMPICSVSKQFTCAVLL	536	0.53	1010
	* ** * *			
Mutant 1	LDTRMPI**S**SVSKQFTCAVLL	64.1	0.053	1210
Mutant 2	LDTRMPI**G**SVSKQFTCAVLL	556	0.80	695
Mutant 3	LDTRMPIC**C**VSKQFTCAVLL	1.08	0.41	2.63
Mutant 4	LDTRMPIC**G**VSKQFTCAVLL	0.0056[a]	-	-
Mutant 5	LDTR**F**PICSVSKQFTCAVLL	633	0.55	1150
Mutant 6	LDTRMPICSVS**N**QFTCAVLL	0.071	0.89	0.080
Mutant 7	LDTRMPICSVSKQFT**S**AVLL	463	0.40	1160

[a] Velocity at 5 mM substrate.

The specific activities of the purified mutant enzymes (V_{max} and K_m values) were compared (Table I). The K_m and V_{max} values of the mutants (except for mutant 4) toward D-alanine-*p*-nitroanilide were determined from double-reciprocal plots. For mutant 1 (Cys60 to Ser) the substitution resulted in an enzyme with a V_{max} value 12% of that of the natural enzyme, although the K_m value was also significantly decreased to 10% of that of the natural enzyme. The V_{max}/K_m value was calculated to be almost the same as that of the wild-type enzyme. For mutant 2 (Cys60 to Gly), substitution with neutral Gly resulted in a lowered V_{max}/K_m value. For mutant 3 (Ser61 to Cys), a substitution at Ser61 with Cys yielded an enzyme with (2×10^2)-fold lower specific activity, while the K_m did not vary so much from that of the native enzyme. For mutant 4 (Ser61 to Gly), the substitution of the Ser61 with Gly yielded an enzyme with only subtle specific activity (0.0056 unit/mg at a substrate concentration of 5.0 mM), which is about 10^5-fold lower than that of the native enzyme. For mutant 5 (Met55 to Phe), substitution at Met55 did not show drastic changes in the kinetic profiles of the enzyme. For mutant 6 (Lys 64 to Asn), substitution at Lys64 into neutral Asn produced an enzyme with drastically lowered activity. The V_{max} value was reduced 10^4-fold, while the K_m value remained in the same order as that of the natural enzyme. For mutant 7 (Cys68 to Ser), substitution at Cys68 caused slightly lowered V_{max} and K_m values. The measurement and evaluation of value of the specificity constant, V_{max}/K_m, suggested that the sites at Ser61 and Lys64 are directly involved in the catalysis of the enzyme: the K_m values are at the same level as in the wild-type enzyme in mutants 3 and 5, while their V_{max} values are significantly lowered. Other mutations (mutants 5 and 7) do not seem to be important in either the binding or catalysis of the enzyme. Noteworthy is the mutation at Cys60 to Ser. Both the V_{max} and K_m values were reduced about 10 times, giving an unaltered V_{max}/K_m value compared to that of the natural enzyme. Thus, Cys60 does not appear to be involved in the catalysis, but in the binding of the substrate.

Effects of typical thiol and serine peptidase inhibitors toward the natural and mutant D-aminopeptidases were examined (Table II). Inhibition patterns by none of the agents varied much for mutants 3–6. The inhibition by PCMB was greatly weakened by the mutant at Cys60 (mutants 1 and 2), which indicates that the inhibition by PCMB was mainly caused by the site of Cys60. The mode of the inhibition by *N*-ethylmaleimide varied among the mutants. Enhancement of in-

```
dd      1  MVSGTVGRGTALGAVLLALLAVPAQAGTAAAADLPAPDDTGLQAVLHTALSQGAPGAMVRVDDNGTIHQL
                                                          .*. *...    *.*.... *
dap     1                                    MSKFDTSALEAFVRHIPQNYKGPGGVVAVVKDGEVVLQH
                                                       . .. .. * ** .***. .*.
bla     1              MFKTTLCALLITASCSTFAAPQQINDIVHRTITPLIEQQKIPGMAVAVIYQGKPYY-F

dd     71  SEGVADRATGRAITTTDRFRVGSVTKSFSAVVLLQLVDEGKLDLDASVNTYLPGLLPDDRITVRQVMSHR
           . * **  *  ..* ..*... **.* *. .***. *.* .* **.....** . . *.* .**.. ...
dap    40  AWGFADLRTRTPMTLDTRMPICSVSKQFTCAVLLDAVGEPEL-LDDALEAYL-DKFEDERPAVRDLCNNQ
           .**.**. .. *.* .* . . **** ** ..  **..  *. *.*. . *   ..... .  .*
bla    58  TWGYADIAKKQPVTQQTLFELGSVSKTFTGVLGGDAIARGEIKLSDPTTKYW-PELTAKQWNGITL----

dd    141  SGLYDYTNDMFAQTVPGFESVRNKVFSYQDLITLSLKHGVTNAPGAAYSYSNTNFVVAGMLIEKLTGHSV
           *** **    .* .*   ..  .  .. *.   *.  ...  .**. ***.*.** . . ***  **...
dap   107  SGLRDY----WALSVLCGADPEGVFLPAQAQSLLRRLKTTHFEPGSHYSYCNGNFRILADLIEAHTGRTL
             * .*     . . *  . *..*  ...  .. .. ... ..**..  * *... ....*   ..* ..
bla   123  LHLATY-----TAGGLPLQVPDEVKSSSDLLRFYQNWQPA-WAPGTQRLYANSSIGLFGALAVKPSGLSF

dd    211  ATEYQNRIFTPLNLTDTFYVHPDTVIPGTHANGYLTPDEAGGALVDSTEQTVSW-AQTGA-AVISSTQDL
           ..   .***.* ... .  . .**.. . . .**   ...  ... .*.. . * ...*  * ...  .
dap   174  VDILSERIFAPAGMKRAELI-SDTALFD-ECTGY--EGDTVRGFLPATNR-IQWMGDAGICASLNDMIAW
            . . .*.*.*  ....              **
bla   187  EQAMQTRVFQPLKLNHTWINVPPAEEKN-YAWGY--REGKAVHVSPGALDAEAYGVKSTIEDMARWVQSN

dd    279  DTFFSALMSGQLMSAAQLAQMQQWTTVNSTQGYGLGLRRRDLSCGISVYGHTGTVQGYYTYAFASKDGQA
           . *..* ....     .*.  * ... . .. **.**. .. . * .. **.*...*
dap   239  EQFIDATRDDESGLYRRLSGPQTFKD-GVAAPYGFGLNLHETG-GKRLTGHGGALRGWRCQRWHCADERL

bla   254  LKPLDINEKTLQQGIQLAQSRYWQTGDMYQGLGWEMLDWPVNPDSIINGSDNKIALAARPVKAITPPTPA

dd    349  HVTALANTSNNVNVLNTMARTLESAFCGKPTTGEAAQRDLLGDHRGAPEDIAPGIARD

dap   307  STIAMFNFEGGASEVAFKLMNIALGVSSSEVSRVEADSAWFGSWLDDETGLVLSLEDAGHGRMKARFGTS

bla   324  VRASWVHKTGATGGFGSYVAFIPEKELGIVMLANKNYPNPARVDAAWQILNALQ

dap   377  PEMMDVVSANEARSAVTTIRRDGETIELVRASENLRLSMKRVKGEAKHDIIGRYHSDELDADLLLVSEGG

dap   447  AIYGAFEGFLGKSDMYPLYSVGSDVWLLPVQRSMDAPSPGEWKLVFRRDDKGEITGLSVGCWLARGVEYR

dap   517  RVQP
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FIGURE 3: Comparison of the amino acid sequences of the *dap* gene from *O. anthropi* SCRC C1-38, carboxypeptidase DD from *Streptomyces* R61, and class C β-lactamase from *E. coli*. The first amino acid sequence (dd) is the sequence of carboxypeptidase DD from *Streptomyces* R61 [sequence size 406 (Duez et al., 1987)]. The second amino acid sequence (dap) is that of D-aminopeptidase from *O. anthropi* [sequence size 520 (this study)]. The third amino acid sequence (bla) is that of class C β-lactamase from *E. coli* K12 [sequence size 377 (Jaurin & Grundström, 1981)]. Symbols: (*) identical residue; (·) similarity of functional group.

hibition by DFP and NEM was observed in mutants 1 and 2, although the reason for this was not clear.

Screening for Other Activities Shown by D-*Aminopeptidase.* At any pH (pH 3–9) and time (up to 6-h incubation) tested, the β-lactam compounds (ampicillin, benzylpenicillin, 6-APA, 7-ACA) remained intact in the presence of D-aminopeptidase (10 units/mL of reaction mixture): no product from the compounds was detected by TLC analyses. D-Alanine, a possible product by the carboxypeptidase DD from N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala and by the transpeptidation reaction between N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala and glycylglycine, could not be detected at any condition tested, even when any of the elements Zn^{2+}, Mn^{2+}, Ca^{2+}, or Mg^{2+} (5 mM) was added to the latter reaction mixture.

Inhibition of the Enzyme by β-Lactam Compounds and N^{α},N^{ϵ}-*Diacetyl-*L-*Lys-*D-*Ala-*D-*Ala.* Because D-aminopeptidase was shown to be structurally related to the penicillin-recognizing enzymes, whether the substrates of this group of enzymes inhibit its enzyme activity was examined. The enzyme was significantly inhibited by β-lactam compounds, such as 6-APA, 7-ACA, benzylpenicillin, and ampicillin. First, we examined the time course of the inhibition. A time-dependent decrease of the enzyme activity, reaching a plateau in 5–10 min, was observed with 6-APA and 7-ACA (data not shown), while the enzyme was inhibited at once with ampicillin or benzylpenicillin. No degradation product has been detected by TLC in the reaction mixtures. The inhibited activities by 6-APA and 7-ACA were gradually restored in a few minutes by more than 10-fold dilution of the reaction mixture by 0.05 M Tris HCl, pH 8.0, or by overnight dialysis against 10 mM potassium phosphate buffer, pH 7.0. The sensitivity of the TLC detection was so high that no more than 0.1–1% of the degradation product could have been detected. However, we have only sought a catalytic degradation of the agents with

			Reference
DAP	O.anthropi	DLRTRTPMTLDTRMPICSVSKQFTCAVLLDAVGEPELL	This study
Class A	S. aureus	KSGKEVKFNSDKRFAYASTSKAINSAILLEQVPYNKLN	Chan (1986)
	K. neumoniae	FAMCSTSKANTVA	Joris et al. (1987)
	E. coli RTEM	SGKILESFRPEERFPMMSTFKVLLCGAVLSRVDAGQEQ	Ambler et al. (1978)
	S. cacaoi	RDGQEITHRADERFAYGSTFKALQAGAILAQVLRDGRE	Lenzini et al. (1987)
	B. licheniformis	GTNRTVAYRPDERFAFASTIKALTVGVLLQQKSIEDLN	Neuberger et al. (1981)
	B. cereus	GTNQTISYRPNERFAFASTYKALAAGVLLQQNSIDSLN	Sloma & Gross (1983)
	S. albus	GSGRTVAYRADELFPMCSVFKTLSSAAVLRDLDRNGEF	Dehottay et al., (1987)
Class C	C. freundii	DIANNHPVTQQTLFELGSVSKTFNGVLGGDRIARGEIK	Lindberg & Normark (1986)
	E. coli K12	LIAKKQPVTQQTLFELGSVSKTFTGVLGGDAIARGEIK	Jaurin & Grundström (1981)
	E. cloacae	DIAANKPVTPQTLFELGSISKTFTGVLGGDAIARGEIS	Galleni et al. (1988))
	P. aeruginosa	VTPETLFEIGIGSVSVSK	Knott-Hunziker et al. (1982)
	S. marcescens	QTGKPITEQTLFEVGSLSK	Joris et al. (1985)
Class D	Staphylococcus	AMLVFDPVRSKKRYSPASTFKIPHTLFALDAGAVRDEF	Dale et al. (1985)
	S. marcescens	SCATNDLARASKEYLPASTFKIPNAIIGLETGVIKNEH	Huovinen et al. (1988)
PBP 1A	E. coli	FNQSKFNRATQALRQVGSNIKPFLYTAAMDKGLTLASM	Broome-Smith et al. (1985)
1B		PQFAGYNRAMQARRSIGSLAKPATYLTALSQPKIYRLN	Broome-Smith et al. (1985)
2		KSGKEVKFNSDKRFAYASTSKAINSAILLEQVPYNKLN	Asoh et al. (1986)
3		PKEAMRNRTITDVFEPGSTVKPMVVMTALQRGVVRENS	Nakamura et al. (1983)
5		SGKVLAEQNADVRRDPASLTKMMTSYVIGQAMKAGKFK	Broome-Smith et al. (1985)
6		SGKVLAEGNADEKLDPASLTKIMTSYVVGQALKADKIK	Broome-Smith et al. (1985)
PBP	S. aureus	DKKEPLLNKFQITTSPGSTQKILTAMIGLNNKTLDDKT	Song et al. (1987)
PBP2	N. gonorrhoeae	DSEQRRNRAVTDMIEPGSAIKPFVIAKALDAGKTDLNE	Spratt (1988)
CPase	Streptomyces	DRATGRAITTTDRFRVGSVTKSFSAVVLLQLVDEGKLD	Duez et al. (1987)
	B. stearothermophilus	TGKILYGKNIDTVLGIASMTKM	Yocum et al. (1980)

FIGURE 4: Partial alignments of the amino acid sequences of D-aminopeptidase and active sites of the penicillin-recognizing enzymes.

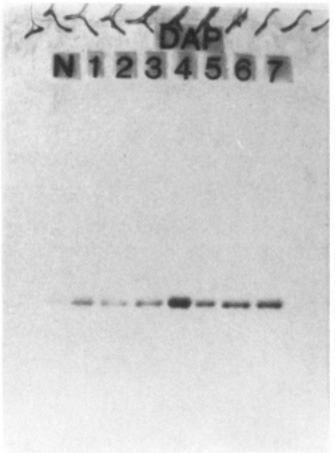

FIGURE 5: SDS gel electrophoresis of the purified D-aminopeptidase mutants. Lane N, native D-aminopeptidase purified from *E. coli* transformant; lanes 1–7, mutant enzymes 1–7, respectively.

Table II: Inhibition of Natural and Mutant D-Aminopeptidases by Several Thiol and Serine Enzyme Inhibitors[a]

	thiol hydrolase inhibitors			serine hydrolase inhibitors	
D-aminopeptidase	DNB[b]	NEM[c]	PCMB[d]	PMSF[e]	DFP[f]
natural	60	56	1.7	96	57
mutant 1, Cys60 → Ser	55	19	83	93	16
mutant 2, Cys60 → Gly	73	15	55	87	6.0
mutant 3, Ser61 → Cys	54	41	2.4	100	50
mutant 5, Met57 → Phe	59	71	1.4	99	74
mutant 6, Lys64 → Asn	70	26	8.6	99	56
mutant 7, Cys68 → Ser	59	39	0.2	99	61

[a] All values are given in percent (%). Reaction mixtures (0.4 mL) containing various concentrations of inhibitors, 250 mM Tris-HCl, pH 8.0, and 0.05 unit of the enzyme were incubated at 30 °C for 30 min, and then the remaining activities were measured with D-alanine-*p*-nitroanilide as substrate. The amounts of the agents in the reaction mixture were as follows: [b] 0.0825 mM; [c] 2.5 mM; [d] 0.0925 mM; [e,f] 1.25 mM. Remaining activities after the treatments are shown.

about 1.7×10^4 times more substrate than the enzyme (10 units/assay = 2.9×10^{-10} mol of the enzyme was used against 5×10^{-6} mol of the substrate). It remained to be studied whether there could be degradation products when the enzyme was used stoichiometrically with respect to the amount of inhibitors, as has been reported with carboxypeptidase DD which yields an acyl-enzyme complex with a longer half-life and products (Frère et al., 1974, 1978; Frère & Joris, 1985).

Table III: Inhibition of the Enzyme by Several β-Lactam Compounds with $(D\text{-Ala})_2$ as a Substrate[a]

inhibitor	type of inhibition	K_i (mM)
7-ACA	competitive	0.1
6-APA	competitive	0.3
ampicillin	competitive	1.9
N-Boc-6-APA	competitive	2.3
N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala	competitive	5.3
benzylpenicillin	competitive	5.9
6-APA-OCH_3	noncompetitive	20
N-Boc-6-APA-OCH_3	no inhibition	
N-Boc-ampicillin-OCH_3	no inhibition	

[a] The inhibition studies were carried out as described in Materials and Methods.

We next investigated the kinetics of the inhibition by the β-lactam compounds and N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala, when D-alanine-*p*-nitroanilide and D-alanine oligomers were used as substrates.

(*A*) *D-Alanine-p-nitroanilide as a Substrate.* Inhibition types were judged with D-alanine-*p*-nitroanilide as a substrate (Cleland, 1963). N^{α},N^{ϵ}-Diacetyl-L-*Lys*-D-Ala-D-Ala acted as a competitive inhibitor with a K_i value of 2.4 mM. 7-ACA (K_i = 2.0 mM) and ampicillin (5.7 mM) were noncompetitive inhibitors, followed by 6-APA (10 mM). Only benzylpenicillin acted as an uncompetitive inhibitor with relatively high K_i value (19 mM). The characteristics of the former three noncompetitive inhibitors are that they all have a β-lactam ring and amino and 2-carboxy groups. Benzylpenicillin has no amino group, which may have resulted in showing a different type of inhibition. The use of the synthetic substrate D-alanine-*p*-nitroanilide resulted in observing various inhibition types; the reason for this could not be explained well.

(*B*) $(D\text{-}Ala)_2$ *as a Substrate.* As summarized in Table III, 7-ACA, 6-APA, ampicillin, *N*-Boc-6-APA, N^{α},N^{ϵ}-diacetyl-

Table IV: Inhibition of the Enzyme by N^{α},N^{ϵ}-Diacetyl-L-Lys-D-Ala-D-Ala with D-Alanine Oligomers as Substrates[a]

substrate	type of inhibition	K_i (mM)
D-alanine-*p*-nitroanilide	competitive	2.4
$(D\text{-Ala})_2$	competitive	5.3[b]
$(D\text{-Ala})_3$	competitive	4.8
$(D\text{-Ala})_4$	competitive	3.2

[a] The conditions were as described in Table III. [b] Value from Table III.

L-Lys-D-Ala-D-Ala, and benzylpenicillin were all good competitive inhibitors of the enzyme, with K_i values ranging from 0.1 to 5.9 mM, when $(D\text{-Ala})_2$ was used as a substrate. The K_i values were smaller than those with D-alanine-*p*-nitroanilide using the same inhibitors. $(D\text{-Ala})_2$ may be more natural than D-alanine-*p*-nitroanilide as a substrate. In this case, a free carboxyl group in the substrate, but not an amino group, appears to be a necessary factor to work as a competitive inhibitor. On the other hand, 6-APA-OCH_3 noncompetitively inhibited the enzyme, with much high K_i value (20 mM). Compounds potected at both of the functional groups, such as *N*-Boc-6-APA-OCH_3 and *N*-Boc-ampicillin-OCH_3, were inert as inhibitors. This result shows that the presence of a carboxyl group is an important factor to be a good inhibitor. Probably, a site recognizing the carboxyl group exists in the enzyme.

(C) Inhibition by N^{α},N^{ϵ}-Diacetyl-L-Lys-D-Ala-D-Ala. More kinetic studies were carried out to determine modes of inhibition by N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala, where D-alanine oligomers were used as substrates, and compared with those with D-alanine-*p*-nitroanilide and $(D\text{-Ala})_2$. N^{α},N^{ϵ}-Diacetyl-L-Lys-D-Ala-D-Ala showed competitive type of inhibition with all of the substrates with relatively lower K_i values (2.4–5.3 mM) (Table IV). The more the degree of polymerization of the D-alanine oligomers, the smaller became the K_i values. The inhibitor N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala structurally resembles the substrate D-alanine oligomers since they all have a D-alanyl-D-alanine moiety and either an amino group or a carboxy group at the same position as β-lactam compounds (Dive et al., 1984; Labischinski et al., 1985). A relatively higher K_i value was observed with N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala (competitive, 5.3 mM) than with 7-ACA (competitive, 0.1 mM) and 6-APA (competitive, 0.3 mM) (Table III), when $(D\text{-Ala})_2$ was used as a substrate. Lack of a free amino group in N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala may have caused its lower affinity with the enzyme.

Discussion

We previously reported the screening, purification, and characterization of a novel aminopeptidase active toward D-amino acid containing peptides, D-amino acid amides, and D-amino acid esters from the bacterium *O. anthropi* SCRC C1-38 (Asano et al., 1989b). In this study, the gene encoding D-aminopeptidase from the bacterium was cloned and sequenced. The overproduction of the enzyme up to 3600-fold per liter of culture as compared with the wild-type strain *O. anthropi* was achieved by a transformant with the *dap* gene placed downstream of the *lac* promoter. The deduced primary structure of the enzyme was unexpectedly similar to that of carboxypeptidase DD, β-lactamases, and penicillin-binding proteins, but not to other aminopeptidases, amide hydrolases, or esterases. Similarities were seen with *Streptomyces* R61 carboxypeptidase DD and class C β-lactamases. A common amino acid sequence Ser-X_{aa}-X_{aa}-Lys was found.

Carboxypeptidase DD, β-lactamases, and penicillin-binding proteins are members of the so-called "penicillin-recognizing enzymes" (Frère et al., 1988; Joris et al., 1986). Soluble carboxypeptidase DD from *Streptomyces* R61 have been intensively studied as a good model of the penicillin-binding proteins. The enzyme catalyzes the cross-linking between the carboxy-terminal D-Ala-D-Ala of a UDP-muramyl pentapeptide (Walsh, 1989) and an amino terminal of glycine oligomer which is linked to diaminopimelic acid (Broome-Smith et al., 1985; Nguyen-Distèche et al., 1986), leaving D-alanine to form a new peptide bond. The enzyme also acts as a carboxypeptidase, hydrolyzing the peptide bond between the D-alanyl-D-alanine moiety in the absence of a nucleophile other than water. Some of the penicillin-binding proteins are membrane-bound and bifunctional with transglucosidase activity, and some only bind to penicillin (Frère et al., 1988). Penicillin and a number of β-lactam compounds are considered to inhibit the transpeptidation reaction to form a penicilloyl-enzyme complex with a long half-life, thereby preventing the synthesis of peptidoglycan, finally causing cell lysis.

β-Lactamases are enzymes which hydrolyze the amide bond of the β-lactam compounds. The deacylation step of the penicilloyl-enzyme complex formed after an nucleophilic attack of the active site Ser to the carbonyl carbon of the amide linkage is much faster than that of a similar enzyme carboxypeptidase DD. Several researchers have proposed classifications of β-lactamases (Ambler, 1980; Medeiros, 1984; Bush, 1989; Matagne et al., 1990), and they are mainly grouped into classes A–D. Ambler first proposed in 1980 to distinguish β-lactamases known at that time into classes A and B, on the basis of their primary structures (Ambler, 1980). Class A enzymes have a common structure with Ser-X_{aa}-X_{aa}-Lys at their active site, while class B enzymes are Zn^{2+}-requiring ones and structurally unrelated to class A enzymes (Hussain et al., 1985). Later, Jaurin and Grundström (1981) proposed to add a new group, class C, and showed that the primary structure of *E. coli* cephalosporinase is different from that of class A enzymes, except for that around the Ser active site. The primary structure or partial sequence around the active sites of the class C β-lactamases have been determined in *Citrobacter freundii* (Lindberg & Normark, 1986), *E. coli* (Jaurin & Grundström, 1981), *Enterobacter cloacae* (Galleni et al., 1988), *Pseudomonas aeruginosa* (Knott-Hunziker et al., 1982), and *Serratia marcescens* (Joris et al., 1985). Another class of enzymes, "D", has been proposed for OXA-2 (Dale et al., 1985) and PSE-2 (Huovinen et al., 1988) β-lactamases. A vast number of reports have appeared to show structural similarities among the class A, C, and D β-lactamases, *Streptomyces* carboxypeptidase DD, and the penicillin-binding proteins (Ambler, 1980; Frère et al., 1985, 1988; Joris et al., 1986). X-ray crystallographic studies have revealed that the three-dimensional structures of the enzymes are similar (Herzberg & Moult, 1987; Kelly et al., 1989). Joris et al. showed by computer alignment of amino acid sequences that the β-lactamases (classes A, C, and D), penicillin-binding domains of the high-M_r penicillin-binding proteins, and *Streptomyces* carboxypeptidase DD are categorized into one superfamily of active-site-serine hydrolases, i.e., "penicillin-recognizing enzymes" (Frère et al., 1985; Joris et al., 1986).

We propose the D-aminopeptidase is a new member of the "penicillin-recognizing enzymes", because of (i) similarities in the primary structure by gene sequencing, (ii) similarities in the reactions catalyzed in water (Asano et al., 1989b, 1991) and organic solvents (Kato et al., 1989, 1990), (iii) the findings obtained by kinetic studies of the mutants generated by the site-directed mutagenesis and (iv) the inhibition by β-lactam

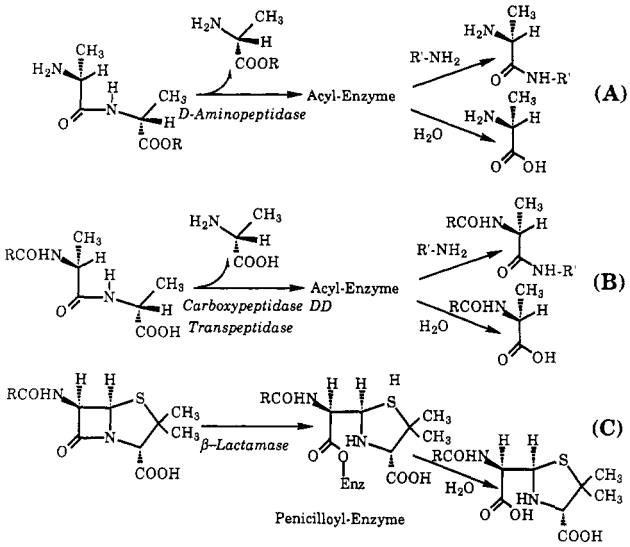

FIGURE 6: Reactions catalyzed by (A) D-aminopeptidase, (B) carboxypeptidase DD, and (C) β-lactamase.

compounds and N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala.

The alignment of the amino acid sequence of D-aminopeptidase showed a similarity with the penicillin-recognizing enzymes, although the former does not act on any of the common substrates of the latter. We would hardly have imagined that an aminopeptidase and a carboxypeptidase would be structurally similar. We previously suggested that D-aminopeptidase would be a thiol peptidase (Asano et al., 1989b). The findings of the site-specific mutagenesis study clearly showed that the amino acid sequences Ser-X_{aa}-X_{aa}-Lys which had been conserved in the penicillin-recognizing enzymes are also essential in exerting the D-aminopeptidase activity. The sites Ser61 and Lys64 are essential in the catalysis, since the V_{max}/K_m values measured with the mutants modified at the positions have been reduced to 0.26–0.008% of those of the natural enzyme, although these K_m values were hardly lowered. Mutant 4 with the inert Gly residue in place of the likely active center Ser61 had a lower activity: 10^5-fold that of the native enzyme. On the other hand, the mutations at other sites (Met 57 and Cys68) did not greatly affect the enzyme activity. The mutations at Cys60, which is adjacent to the likely active center Ser61, gave notable effects on the kinetic profiles, which shows that the residue is important in the binding of the substrate. The substitutions at Cys60 to Ser and Gly produced mutant D-aminopeptidases with slightly altered V_{max}/K_m values. They were tolerant to inhibition by PCMB, which suggests that the inhibition by PCMB of the native enzyme would have been caused by the steric hindrance by a mercaptide bond formation between Cys60 of the enzyme and PCMB. Lowered activities have been detected with β-lactamase after substitutions of the active site Ser with Cys (Sigal et al., 1984) and Lys with Arg (Madgwick & Waley, 1987; Tsukamoto et al., 1990), respectively. The structure of PCMB-sensitive β-lactamases has been reported for the class A enzymes from *Streptomyces albus* G (Dehottay et al., 1987) and *Klebsiella pneumoniae* (Joris et al., 1987), which together with D-aminopeptidase have a Cys residue next to the active site Ser: Cys-Ser-X_{aa}-X_{aa}-Lys. The studies on the inhibition of the enzyme by several β-lactam compounds revealed that the enzyme is actually inhibited with K_i values at a physiological concentration.

Figure 6 shows similarities of the reactions catalyzed by (A) D-aminopeptidase, (B) carboxypeptidase DD, and (C) β-lactamases. All three enzymes catalyze the hydrolytic cleavage of the amide bond between D-amino acids or the same configuration. The former two catalyze the transpeptidation reaction; carboxypeptidase DD catalyzes the cross-linkage of the peptidoglycan, and D-aminopeptidase catalyzes the transpeptidation and aminolysis of D-amino acid ester and amides, respectively, in water and organic solvents. The aminolysis reaction catalyzed by D-aminopeptidase in butyl acetate is efficient (k_{cat}: 7700/min) (Kato et al., 1989). The transpeptidation reaction can also be catalyzed in water, although the product D-alanyl-3-aminopentane amide was easily hydrolyzed in water (Kato et al., 1989). Inheritance of the carboxypeptidase-like tertiary structure in the D-aminopeptidase would have made the transpeptidation reaction in organic solvents possible. Carboxypeptidase DD from *Streptomyces* K15 behaves as a good transpeptidase with N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala and a nucleophile (Walsh, 1989). They all require the configuration of the substrate peptide which has a D-amino acid configuration.

We showed that D-aminopeptidase has sites similar to those of carboxypeptidase DD and β-lactamases, employing mutants altered at some sites around the conserved region. Functionally, D-aminopeptidase, carboxypeptidase DD (a penicillin-binding protein), and β-lactamase have a common feature in that they utilize peptides containing D-amino acid as substrates and the former two catalyze transpeptidation reactions with substrates containing D-alanyl-D-alanine moieties, although D-aminopeptidase does not act on any substrates which carboxypeptidase DD and β-lactamase do. Furthermore, we predicted from a structural and functional viewpoint that the substrates or substrate analogues of β-lactamases inhibit D-aminopeptidase and showed that the enzyme was actually inhibited by them. When D-alanine-*p*-nitrianilide was used as a substrate, N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala competitively inhibited the enzyme, while benzylpenicillin did uncompetitively and ampicillin, 6-APA, and 7-ACA did noncompetitively. When the D-alanine dimer was used as a substrate, β-lactam compounds such as 7-ACA, 6-APA, ampicillin, N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala, and benzylpenicillin competitively inhibited the enzyme, while 6-APA-OCH_3 did noncompetitively. When D-alanine-*p*-nitroanilide and D-alanine oligomers (n = 2–4) were used as substrates, N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala acted as a competitive inhibitor. Among the inhibitors in general, it is noteworthy that 7-ACA showed the lowest K_i values and N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala acted as an competitive inhibitor against various substrates. These results coincide well with the higher similarities of the enzyme with *Streptomyces* carboxypeptidase DD (27%) (Duez et al., 1987 and *E. coli* cephalosporinase (23%) (Jaurin & Grundström, 1981), the substrates of which are cephalosporin and N^{α},N^{ϵ}-diacetyl-L-Lys-D-Ala-D-Ala, respectively. From these results, the enzyme may have a binding pocket at the active center to accommodate a D-Ala-D-Ala moiety, which resembles the tertiary structure of the β-lactam ring of the antibiotics. An amino group binding site may be located in the enzyme to recognize substrates with a free amino group of D-amino acid derivatives, as evidenced by the following results: (i) The enzyme preferentially acts on peptides with a D-amino acid at the N-terminus (Asano et al., 1989b). (ii) 7-ACA and 6-APA act as competitive inhibitors with low K_i values when $(D\text{-Ala})_2$ was used as a substrate. (iii) K_i values became much higher when the amino groups of the inhibitors were blocked with substituents such as D-phenylglycyl, BOC, and N^{α},N^{ϵ}-diacetyl-L-lysyl, although the inhibition types were still competitive. Furthermore, a carboxylate binding site in the enzyme may be also important, since the agents modified

as methyl esters were inert or a noncompetitive inhibitor with a high K_i value.

We thus propose that the D-aminopeptidase is a new member of the "penicillin-recognizing enzymes". This is evidence against the discussion that the β-lactamases are evolved from the penicillin-binding proteins (Nicholas & Strominger, 1988). We showed the existence of a third enzyme with a similar structure, which does not appear to be a selective target of the β-lactam antibiotics. The biological role of D-aminopeptidase is still unknown. The substrate specificity and kinetic studies showed that the enzyme prefers D-alanine-containing peptides rather than simple D-amino acid amides (Asano et al., 1989). The substrates D-alanyl-D-alanine (Nguyen-Distèche et al., 1986) and D-alanylglycylglycine (Manabe, 1986) occur naturally in the rice plant and in peptidoglycan, respectively. The enzyme may have evolved to use the natural substrates, or other unknown functions may exist. There might exist an unknown endopeptidase which hydrolyzes peptidoglycan to yield the peptide with D-alanine at the N-terminus. D-Aminopeptidase probably is the first example of an aminopeptidase with Ser at the active center, since other aminopeptidases are divalent cation-dependent or thiol enzymes (DeLange & Smith, 1971).

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References

Ambler, R. P. (1980) *Philos. Trans. R. Soc. London, B 289*, 321–331.

Ambler, R. P., & Scott, G. K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3732–3736.

Asano, Y., Fujishiro, K., Tani, Y., & Yamada, H. (1982) *Agric. Biol. Chem. 46*, 1165–1174.

Asano, Y., Nakazawa, A., & Endo, K. (1987) *J. Biol. Chem. 262*, 10346–10354.

Asano, Y., Nakazawa, A., Kato, Y., & Kondo, K. (1989a) *Angew. Chem. 101*, 511–512.

Asano, Y., Nakazawa, A., Kato, Y., & Kondo, K. (1989b) *J. Biol. Chem. 264*, 14233–14239.

Asano, Y., Mori, T., Hanamoto, S., Kato, Y., & Nakazawa, A. (1989c) *Biochem. Biophys. Res. Commun. 162*, 470–474.

Asano, Y., Yamaguchi, K., & Kondo, K. (1989d) *J. Bacteriol. 171*, 4466–4471.

Asano, Y., Yamada, A., Kato, Y., Yamaguchi, K., Hibino, Y., Hirai, K., & Kondo, K. (1990) *J. Org. Chem. 21*, 5567–5571.

Asano, Y., Kishino, K., Yamada, A., Hanamoto, S., & Kondo, K. (1991) *Recl. Trav. Chim. Pays-Bas 110*, 206–208.

Asoh, S., Matsuzawa, H., Ishino, F., Strominger, J. L., Matsuhashi, M., & Ohta, T. (1986) *Eur. J. Biochem. 160*, 231–238.

Birnboim, H. C., & Doly, J. (1979) *Nucleic Acids Res. 7*, 1513–1523.

Brenner, S. (1988) *Nature 334*, 528–530.

Broome-Smith, J. K., Edelman, A., Yousif, S., & Spratt, B. G. (1985) *Eur. J. Biochem. 147*, 437–446.

Broome-Smith, J. K., Ioannidis, I., Edelman, A., & Spratt, B. G. (1988) *Nucleic Acids Res. 16*, 1617.

Bush, K. (1989) *Antimicrob. Agents Chemother. 33*, 259–263.

Chan, P. T. (1986) *Nucleic Acids Res. 14*, 5940.

Cleland, W. W. (1971) *The Enzymes* (Boyer, P. D., Ed.) Vol. 2, pp 1–43, Academic Press, New York.

Dale, J. W., Godwin, D., Mossakowska, D., Stephenson, P., & Wall, S. (1985) *FEBS Lett. 191*, 39–44.

Davis, L. G., Dibner, M. D., & Battey, J. F. (1986) *Basic Methods in Molecular Biology*, Elsevier, New York, NY.

Dehottay, P., Dusart, J., De Meester, F., Joris, B., Van Beeumen, J., Erpicum, T., Frère, J.-M., & Ghuysen, J.-M. (1987) *Eur. J. Biochem. 166*, 345–350.

DeLange, R. J., & Smith, E. L. (1971) *The Enzymes* (Boyer, P. D., Ed.) Vol. 3, pp 81–118, Academic Press, New York.

Dive, G., Peeters, D., Leroy, G., & Ghuysen, J. M. (1984) *J. Mol. Struct. 107*, 117–126.

Duez, C., Piron-Fraipont, C., Joris, B., Dusart, J., Urdea, M. S., Martial, J. A., Frère, J.-M., & Ghuysen, J.-M. (1987) *Eur. J. Biochem. 162*, 509–518.

Frère, J.-M., & Joris, B. (1985) *CRC Crit. Rev. Microbiol. 11*, 299–396.

Frère, J.-M., Leyh-Bouille, M., Ghuysen, J.-M., & Perkins, H. R. (1974) *Eur. J. Biochem. 50*, 203–214.

Frère, J.-M., Leyh-Bouille, M., Ghuysen, J.-M., Nieto, M., & Perkins, H. R. (1976) in *Methods in Enzymology* (Lorando, L., Ed.) Vol. 45, pp 610–636, Adademic Press, New York.

Frère, J.-M., Ghuysen, J.-M., & De Graeve, J. (1978) *FEBS Lett. 88*, 147–150.

Frère, J.-M., Joris, B., Dideberg, O., Charlier, P., & Ghuysen, J.-M. (1988) *Biochem. Soc. Trans. 16*, 934–938.

Galleni, M., Lindberg, F., Normark, S., Cole, S., Honore, N., Joris, B., & Frère, J.-M. (1988) *Biochem. J. 250*, 753–760.

Hanahan, D. (1983) *J. Mol. Biol. 166*, 557–580.

Heinkoff, S. (1984) *Gene 28*, 351–359.

Herzberg, O., & Moult, J. (1987) *Science 236*, 694–701.

Higaki, J. N., Evnin, L. B., & Craik, C. S. (1989) *Biochemistry 28*, 9256–9263.

Holmes, B., Popoff, M., Kiredjian, M., & Kersters, K. (1988) *Int. J. Syst. Bacteriol. 38*, 406–416.

Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J., & Hood, L. E. (1983) *Methods Enzymol. 91*, 399–434.

Huovinen, P., Huovinen, S., & Jacoby, G. A. (1988) *Antimicrob. Agents Chemother, 32*, 134–136.

Hussain, M., Carlino, A., Madonna, J., & Lampen, J. O. (1985) *J. Bacteriol. 164*, 223–229.

Jaurin, B., & Grundström, T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4897–4901.

Joris, B., De Meester, F., Galleni, M., Reckinger, G., Coyette, J., & Frère, J.-M. (1985) *Biochem. J. 228*, 241–248.

Joris, B., De Messer, F., Galleni, M., Masson, S., Dusart, J., Frère, J.-M., Van Beeumen, J., Bush, K., & Sykes, R. (1986) *Biochem. J. 239*, 581–586.

Joris, B., De Meester, F., Galleni, M., Frère, J.-M., & Van Beeumen, J. (1987) *Biochem. J. 243*, 561–567.

Kato, Y., Asano, Y., Nakazawa, A., & Kondo, K. (1989) *Tetrahedron 45*, 5743–5754.

Kato, Y., Asano, Y., Nakazawa, A., & Kondo, K. (1990) *Biocatalysis 3*, 207–215.

Kelly, J. A., Knox, J. R., Zhao, H., Frère, J.-M., & Ghuysen, J.-M. (1989) *J. Mol. Biol. 209*, 281–295.

Knott-Hunziker, V., Petursson, S., Jayatilake, G. S., Waley, S. G., Jaurin, B., & Grundström, T. (1982) *Biochem. J. 201*, 621–627.

Korn, L. J., Queen, C. L., & Wegman, M. N. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4401–4405.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488-492.
Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367-382.
Labischinski, H., Barnickel, G., Naumann, D., Ronspeck, W., & Bradaczek, H. (1985) *Biopolymers 24*, 2087-2112.
Laemmli, U. K. (1970) *Nature 227*, 680-685.
Lenzini, M. V., Ishihara, H., Dusart, J., Ogawa, H., Joris, B., Van Beeumen, J., Frère, J.-M., & Ghuysen, J.-M. (1987) *FEMS Microbiol. Lett. 49*, 371-376.
Lindberg, F., & Normark, S. (1986) *Eur. J. Biochem. 156*, 441-445.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Madgwick, P. J., & Waley, S. G. (1987) *Biochem. J. 248*, 657-662.
Manabe, H. (1986) *Phytochemistry 25*, 2233-2235.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Matagne, A., Misselyn-Bauduin, A.-M., Joris, B., Erpicum, T., Granier, B., & Frère, J.-M. (1990) *Biochem. J. 265*, 131-146.
Medeiros, A. A. (1984) *Br. Med. Bull. 40*, 18-27.
Nakamura, M., Maruyama, I. N., Soma, M., Kato, J., Suzuki, H., & Horota, Y. (1983) *Mol. Gen. Genet. 191*, 1-9.
Neugebauer, K., Sprengel, R., & Schaller, H. (1981) *Nucleic Acids Res. 9*, 2577-2588.
Nguyen-Distèche, M., Leyh-Bouille, M., Pirlot, S., Frère, J.-M., & Ghuysen, J.-M. (1986) *Biochem. J. 235*, 167-176.
Nicholas, R. A., & Strominger, J. L. (1988) *Rev. Infect. Dis. 10*, 733-742.
Rodriguez, R. L., & Tait, R. C. (1983) *Recombinant DNA Techniques: An Introduction*, The Benjamin/Cummings Publishing Co., Menlo Park, CA.
Sanger, F., Nickeln, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Schleifer, K. H., & Kandler, O. (1972) *Bacteriol. Rev. 36*, 407-477.
Shine, J., & Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1342-1346.
Sigal, I. S., DeGaro, W. F., Thomas, B. J., Petteway, S. R., Jr. (1984) *J. Biol. Chem. 259*, 5327-5332.
Sloma, A., & Gross, M. (1983) *Nucleic Acids Res. 11*, 4997-5004.
Song, M. D., Wachi, M., Doi, M., Ishino, F., & Matsuhashi, M. (1987) *FEBS Lett. 221*, 167-171.
Spratt, B. G. (1988) *Nature 332*, 173-176.
Stormo, G. D., Schneider, T. D., & Gold, L. M. (1982) *Nucleic Acids Res.* 2971-2996.
Tsukamoto, K., Tachibana, K., Yamazaki, N., Ishii, Y., Ujiie, Y., Nishida, N., & Sawai, T. (1990) *Eur. J. Biochem. 118*, 15-22.
Vieira, J., & Messing, J. (1982) *Gene 19*, 259-268.
Vieira, J., & Messing, J. (1987) *Methods Enzymol. 153*, 3-11.
Walsh, C. T. (1989) *J. Biol. Chem. 264*, 2393-2396.
Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103-119.
Yocum, R. R., Rasmussen, J. R., & Strominger, J. L. (1980) *J. Biol. Chem. 255*, 3977-3986.

Conversion of the Noncooperative *Bacillus subtilis* Aspartate Transcarbamoylase into a Cooperative Enzyme by a Single Amino Acid Substitution†

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ABSTRACT: Allosteric enzymes are part of a unique class of enzymes which regulate metabolic pathways. On the molecular level, allosteric regulation is the result of interactions between discrete binding sites on the enzyme. In order to accommodate these multiple binding sites, allosteric enzymes have evolved with oligomeric quaternary structures. However, only a few oligomeric enzymes are known to have regulatory interactions between binding sites. Is regulatory activity an inherent property of oligomeric enzymes? The trimeric *Bacillus subtilis* aspartate transcarbamoylase catalyzes the first committed step of the pyrimidine biosynthetic pathway and is not known to be a regulatory enzyme. When an alanine residue is substituted for the active-site residue Arg-99 by site-specific mutagenesis, the regulatory activity of homotropic substrate cooperativity (Hill coefficient of 1.5) is observed in the resulting mutant enzyme. These results suggest that homotropic regulation may have evolved by a relatively small number of mutations to an oligomeric enzyme.

In the mechanism of allosteric regulation, the overall throughput or rate of a metabolic pathway is limited by the velocity of a key regulatory enzyme. The activity of this enzyme is in turn affected by two types of interactions between discrete binding sites: first, heterotropic interactions between active sites and allosteric binding sites, in which the binding of allosteric molecules can either enhance or diminish activity; second, homotropic interactions between active sites, in which cooperative substrate binding causes a sigmoidal dependence of activity upon substrate concentration. Two microscopic equilibrium models are commonly used to explain the physical basis of homotropic interactions in enzymes (Monod et al., 1965; Koshland et al., 1966). In these models, the enzyme exists in at least two conformations, which differ in catalytic

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