A Novel Metal-activated Pyridoxal Enzyme with a Unique Primary Structure, Low Specificity d-Threonine Aldolase from *Arthrobacter* sp. Strain DK-38

MOLECULAR CLONING AND COFACTOR CHARACTERIZATION *

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The gene encoding low specificity *d*-threonine aldolase, catalyzing the interconversion of *d*-threonine/allo-*d*-threonine and glycine plus acetaldehyde, was cloned from the chromosomal DNA of *Arthrobacter* sp. strain DK-38. The gene contains an open reading frame consisting of 1,140 nucleotides corresponding to 379 amino acid residues. The enzyme was overproduced in recombinant *Escherichia coli* cells and purified to homogeneity by ammonium sulfate fractionation and three-column chromatography steps. The recombinant aldolase was identified as a pyridoxal enzyme with the capacity of binding 1 mol of pyridoxal 5′-phosphate per mol of subunit, and Lys59 of the enzyme was determined to be the cofactor binding site by chemical modification with NaBH4. In addition, Mn2+ ion was demonstrated to be an activator of the enzyme, although the purified enzyme contained no detectable metal ions. Equilibrium dialysis and atomic absorption studies revealed that the recombinant enzyme could bind 1 mol of Mn2+ ion per mol of subunit. Remarkably, the predicted amino acid sequence of the enzyme showed no significant similarity to those of the currently known pyridoxal 5′-phosphate-dependent enzymes, indicating that low specificity *d*-threonine aldolase is a new pyridoxal enzyme with a unique primary structure. Taken together, low specificity *d*-threonine aldolase from *Arthrobacter* sp. strain DK-38, with a unique primary structure, is a novel metal-activated pyridoxal enzyme.

The bioorganic synthesis of *β*-hydroxy-*α*-amino acids attracts a great deal of attention because of their potential application as chiral building blocks for the synthesis of biologically active molecules (1–5). A variety of *β*-hydroxy-*α*-amino acids is present in complex natural compounds with interesting biological properties. 3,4,5-Trihydroxy-*l*-aminopentanoic acid is a key component of polyoxins (1). 4-Hydroxy-*l*-threonine, for example, is a precursor of rizobitoxine, a potent inhibitor of pyridoxal 5′-phosphate (PLP)9-dependent enzymes (1). *D*-isomers are also biologically significant, because they not only exist in mature mammals (6) but are also constituents of a range of antibiotics, for example, Fusaricidin (7) and Viscosin (8).

Threonine aldolase (TA) (EC 4.1.2.5), which catalyzes the reversible interconversion of certain *β*-hydroxy-*α*-amino acids and glycine plus aldehydes, has been shown to be useful for the biosynthesis of *β*-hydroxy-*α*-amino acids (1–5). The enzyme appears to fall into two types, *l*-type and *d*-type, on the basis of its stereospecificity of the cleavage reaction toward the *α*-carbon of threonine. *l*-Type TA, acting on *l*- and/or *l*-allo-threonine, is further divided into three groups based on its stereospecificity toward the *β*-carbon of threonine as follows: (i) *l*-allo-TA is specific to *l*-allo-threonine; (ii) *l*-TA acts only on *l*-threonine; and (iii) low specificity *l*-TA can use both *l*-threonine and *l*-allo-threonine as substrates. All of the three *l*-type enzymes have been found to exist in nature. *l*-TA was partially purified from *Clostridium pasteurianum* (9); *l*-allo-TA was from *Aeromonas jandaei* (10), and three low specificity *l*-TAs from *Candida humicola* (11, 12), *Saccharomyces cerevisiae* (13), and *Pseudomonas* sp. strain (14) were purified and extensively characterized. The genes encoding for *l*-allo-TA of *A. jandaei* and low specificity *l*-TAs of *S. cerevisiae* and *Pseudomonas* sp. strain have been cloned and sequenced (13–15). Likewise, *d*-type TA, acting on *d*-threonine and/or *d*-allo-threonine, might include *d*-allo-TA, *d*-TA, and low specificity *d*-TA. However, only low specificity *d*-TA activity was found from 3 out of 2,000 strains examined (16). Low specificity *d*-TA was previously purified from *Arthrobacter* sp. strain DK-38, and the enzyme was shown to have a monomeric structure and to require unusually both PLP and divalent cations for its catalytic activity (16). Due to little available purified enzyme, the identification of the cofactors was not performed.

The present paper describes cloning and sequencing of the *dtaAS* gene encoding the low specificity *d*-TA from *Arthrobacter* sp. strain DK-38, the expression of the gene in *Escherichia coli* cells, and further characterization of the recombinant enzyme. Our data showed that the low specificity *d*-TA is a novel metal-activated pyridoxal enzyme. Evidence is also presented that low specificity *d*-TA has a unique primary structure, probably representing a new family of pyridoxal enzyme.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank TM/ EBI Data Bank with accession number(s) AB010956.

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* The abbreviations used are: PLP, pyridoxal 5′-phosphate; TA, threonine aldolase; IPTG, isopropyl-*β*-d-thiogalactoside; LB, Luria Bertani; bp, base pair(s); kb, kilobase; PCR, polymerase chain reaction; ORF, open reading frame.
EXPERIMENTAL PROCEDURES

Materials

Butyl-Toyopearl 650M and DEAE-Toyopearl 650M were purchased from Tosoh (Tokyo, Japan); Gigapite was obtained from Toagosei (Tokyo, Japan). Pyridoxal-5'-phosphate (PLP) and pyridoxine-5'-phosphate monohydrochloride were bought from Nacalai (Kyoto, Japan). nv-threo-β-Phe-lyserine and nv-threo-β-(3,4-dihydroxyphenyl)serine were purchased from Sigma. nv-erythro-β-Phe-lyserine was a generous gift from Hideyuki Hayashi and Hiroyuki Kagamiyama, Department of Biochemistry, Osaka Medical College, Osaka, Japan. DL-threo-pyroglutamate was a generous gift from Hideyuki Hayashi and Hiroyuki Kagamiyama, Department of Biochemistry, Osaka Medical College, Osaka, Japan.

Bacterial Strains, Plasmids, and Culture Conditions

Arthrobacter sp. strain DK-38 was used as the source of chromosomal DNA (16). E. coli XL1-Blue MRF (recA1 thi endA1 supE44 gyrA46 relA1 hisdR17 lacI517) (Takara Shuzo, Kyoto, Japan) was used as a cloning vector. pKK 223-3 (Amersham Pharmacia Biotech, Uppsala, Sweden) was used as a vector for overexpression of the dtaAS gene. Arthrobacter sp. strain DK-38 was grown in a medium comprising 1% peptone, 0.5% yeast extract, and 1% Biochemistry, Osaka Medical College, Osaka, Japan. nv-threo-β-Phe-lyserine and nv-erythro-β-(3,4-dihydroxyphenyl)serine were prepared according to the method of Ohashi et al. (17). The other chemicals were all analytical grade reagents.

Cloning of the dtaAS Gene

Two oligonucleotide primers were purchased from Amersham Pharmacia Biotech (Tokyo, Japan), each with additional restricted site (underlined in the following sequences) added to the 5’ end to facilitate cloning of the amplified product: primer I, 5’-CGCAAGCTTATGGCAAGGACCGGAGTGCA-3’; and primer II, 5’-CGGATCCGCGCGAGCGGAGC-3’. Degenerate positions are indicated by “S” for C or G, “R” for A or G, and “N” for all bases. Primers I and II were based upon the NH2-terminal amino acid sequence of the wild-type low specificity d-threonine aldolase from Arthrobacter sp. strain DK-38. PCR amplification was performed in a 50-μl reaction mixture containing 5 μl of MeSO4, 10 mM Tris-HCl (pH 8.85), 25 mM KCl, 5 mM (NH4)2SO4, 2 mM MgSO4, 0.1 mM deoxynucleotide triphosphate, 20 pmol of each primer, 1 μg of the genomic DNA, and 0.5 units of PWO DNA polymerase (Boehringer Mannheim, Germany) at 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min for a total of 30 cycles. The amplified product was digested with EcoRI and HindIII, separated by agarose gel electrophoresis, and then purified with a GeneClean kit. The amplified DNA of approximately 1.1 kb, which contained the complete coding sequences of the dtaAS gene, was inserted into the NcoI site downstream of the tac promoter in pKK223-3 and then used to transform E. coli XL1-Blue MRF cells. The constructed plasmid was designated pKDTA.

Purification of the Recombinant Low Specificity d-TA

All enzyme purification operations were carried out at 0–4 °C. Unless otherwise noted, 50 mM Tris-HCl (pH 7.4) containing 10 μM PLP was used as the buffer throughout the purification procedures.

Step 1, Preparation of Cell Extract—Cells of the E. coli transformant harboring plasmid pKDTA were grown aerobically at 37 °C for 16 h in 12 liters of LB medium containing 0.1 mg/ml ampicillin and 0.2 mM IPTG. The cells were harvested and rinsed with buffer. After being suspended in 200 ml of buffer, the cells were disrupted by ultrasonic oscillation at 4 °C for 20 min with a model 201M ultrasonic oscillator (Kubota, Tokyo, Japan). The cell debris was removed by centrifugation at 25,000 × g for 30 min.

Step 2, Ammonium Sulfate Fractionation—the supernatant solution was brought to 20% saturation with ammonium sulfate and centrifuged at 25,000 × g for 30 min. Ammonium sulfate was added to the supernatant solution to 50% saturation. The precipitate collected by centrifugation was dissolved in buffer, and the enzyme solution was dialyzed against 100 volumes of buffer.

Step 3, Butyl-Toyopearl Column Chromatography—the enzyme solution, brought to 20% saturation with ammonium sulfate, was applied to a Butyl-Toyopearl 650M column (5.0 × 40 cm). Elution was carried out with a 2,400-ml linear gradient of 20 to 0% saturated ammonium sulfate in buffer at a flow rate of 5 ml/min. The fractions with threonine aldolase activity were pooled and concentrated by ultrafiltration with a Centriprep-30 apparatus (Amicon, Inc., Beverly, MA).

Step 4, DEAE-Toyopearl Column Chromatography—the enzyme solution was dialyzed against 1,000 volumes of buffer and applied to a DEAE-Toyopearl 650x column (2.5 × 20 cm) equilibrated with buffer. After the column was thoroughly washed with the buffer containing 50 mM NaCl, linear gradient elution was performed with a buffer supplemented with NaCl by increasing the concentration from 50 to 200 mM. The flow rate was maintained at 5 ml/min. The fractions with d-TA activity were pooled and concentrated by ultrafiltration with a Centriprep-30 apparatus.

Amino Acid Sequencing

Amino acid sequence was determined by the Edman degradation procedure with a model 476A protein sequencer (Perkin-Elmer) or a model PPSQ-10 protein sequencer (Shimadzu, Kyoto, Japan).

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**Low Specificity d-Threonine Aldolase**

Enzyme Assay

Threonine aldolase activity was measured spectrophotometrically at 340 nm by coupling the reduction of acetaldehyde (oxidation of NADH) with yeast alcohol dehydrogenase (Wako, Osaka, Japan). The assay mixture comprised 100 μmol of Hepes-NaOH buffer (pH 8.0), 50 μmol of d-threonine, 0.05 μmol of PLP, 0.1 μmol of MnCl₂, 0.2 μmol of NADH, 30 units of yeast alcohol dehydrogenase, and appropriate amounts of the enzyme in a final volume of 1 ml. One unit of the enzyme is the amount of enzyme that catalyzed the formation of 1 μmol of acetaldehyde (1 μmol of NADH oxidized) per min at 30 °C; the molar extinction coefficient of NADH is 6.2 × 10⁵ M⁻¹ cm⁻¹. For a qualitative analysis, threonine aldolase activity was also assayed as follows: the reaction mixture comprised 10 μmol of d-threonine, 0.01 μmol of PLP, 0.02 μmol of MnCl₂, 20 μmol of Hepes-NaOH buffer (pH 8.0), and the enzyme in a total volume of 1 ml. The reaction was carried out at 30 °C for 10 min and was terminated by the addition of 50 μl of 30% trichloroacetic acid. The released acetaldehyde was measured spectrophotometrically according to the method of Pax et al. (22). The aldolase activities toward phenylserine, β-3,4-dihydroxyphenylserine, and β-3,4-methylenedioxophenylserine were measured spectrophotometrically at 278, 350, and 320 nm, respectively, with molar extinction coefficients of 1.4 × 10⁵ M⁻¹ cm⁻¹ for benzaldehyde, 8.9 × 10³ M⁻¹ cm⁻¹ for protocatechualdehyde, and 17.6 × 10³ M⁻¹ cm⁻¹ for piperonal.

**Determination of Protein**

The concentration of the enzyme was determined spectrophotometrically by using a molar extinction coefficient ε₂₈₀ = 29,927 M⁻¹ cm⁻¹ (λ = 280 nm, ε₈₈₀ = 1.34) at 278 nm and pH 7.4 for the holoenzyme and ε₂₈₀ = 27,562 M⁻¹ cm⁻¹ (λ = 280 nm, ε₈₈₀ = 1.45) at 278 nm for the apoenzyme, where the ε₈₈₀ values were determined by the method of Edelhoch and co-workers (23, 24), and the contributions of tryptophan, tyrosine, and cystine to the ε₈₈₀ values in 6 ml guanidine hydrochloride were calculated on the basis of 3 tryptophan, 7 tyrosine, and 8 cystine residues and 5565, 1395, and 140 M⁻¹ cm⁻¹ for the three residues, respectively.

**Chromatographic Optical Resolution of Amino Acid Stereosomers**

The isomers of β-phenylserine and β-(3,4-methylenedioxyphenyl)serine were analyzed by high performance liquid chromatography as follows: column, Siumichiral OA-5000 (4.6 × 150 mm) (Sumitomo, Tokyo, Japan); solvent, 2 ml copper sulfate containing 15% methanol; flow rate, 1.0 ml/min; detection, 254 nm; and temperature, 30 °C. The isomers of β-3,4-dihydroxyphenylserine were also analyzed by high performance liquid chromatography as follows: column, Crownpak CR (4 × 150 mm) (Daicel, Osaka, Japan); solvent, distilled water adjusted to pH 7.0 with perchloric acid; flow rate, 0.4 ml/min; detection, 220 nm; and temperature, 4 °C.

**Spectrophotometric Measurements**

The absorption spectrum of the enzyme was measured at 20 °C with 20 mM potassium phosphate buffer (pH 7.4) by a Hitachi model U-3210 spectrophotometer (Hitachi, Tokyo, Japan).

**PLP Content**

The PLP content of the enzyme was determined according to the method of Wada and Snell (25).

**Isolation of Pyridoxyl Peptide**

The holoenzyme (10 mg in 200 μl of 20 mM potassium phosphate buffer (pH 7.4)) was reduced at 0 °C by the addition of about 0.2 mg of solid NaBH₄. To protect the label against photo-destruction, all the tubes used in the following experiment were covered with aluminum foil. After standing at 0 °C for about 30 min, the enzyme protein was precipitated with 2% trichloroacetic acid. The precipitates were dissolved in 100 μl of 50 mM Tris-HCl buffer (pH 8.5) containing 6 ml guanidine HCl and 0.5 ml dithiothreitol and treated at 37 °C for 1 h with 2 ml iodooacetate. The reduced and carboxymethylated protein was extensively dialyzed against 5 ml HCl. The precipitates formed during dialysis were collected by centrifugation, washed twice with water, and suspended in 500 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 2.5 ml of 1:1-diolysamido-2-phenyl ethyl chloromethyl ketone-treated trypsin (Worthington) and subsequently digested at 37 °C for 12 h. The resultant peptide fragments were applied to a C₁₄ column (4.6 × 150 mm; Tosoh, Tokyo, Japan) and eluted with 0.05% trifluoroacetic acid for 10 min, followed by a linear gradient of 0–80% acetonitrile in 0.05% trifluoroacetic acid over 60 min at a flow rate of 1.0 ml/min. The elution was monitored daily by absorbance at 215 and 330 nm. The peptide, which had strong absorbance at 330 nm, was further purified by high pressure liquid chromatography under chromatographic conditions similar to those previously described, except a directly linear gradient of 5–50% acetonitrile was employed. The fractions which showed absorbance at 330 nm were pooled and subjected to sequence analysis.

**Generation of Metal-free Enzyme**

Two hundred micromoles of recombinant low specificity d-TA was dialyzed against 50 mM Tris-HCl buffer (pH 7.4) containing 10 μM PLP and 10 mM EDTA for 12 h at 4 °C. The enzyme solution was further dialyzed twice against the same buffer without EDTA for 12 h at 4 °C to remove EDTA.

**Equilibrium Dialysis Study**

Ten nanomoles of the metal-free enzyme was dialyzed twice against 1 liter of 50 mM Tris-HCl buffer (pH 7.4) containing 10 μM PLP and various concentrations of MnCl₂ (0–50 μM) for 12 h at 4 °C. The metal concentrations, both inside and outside the dialysis bag, were then determined as described below.

**Atomic Absorption Measurement**

The metal ion concentration was determined with a mode FLA-1000 atomic absorption spectrometer (Nippon Jarrell-Ash, Uji, Japan).

**RESULTS**

**Cloning of the dtaAS Gene**—The primers used for cloning of the dtaAS gene by PCR were based on the NH₂-terminal amino acid sequence of the purified low specificity d-TA from Arthrobacter sp. strain DK-38. PCR with the primers and Arthrobacter chromosomal DNA as the template yielded an amplified band of 90 bp. Only this band was constantly amplified under different PCR conditions. The amplified DNA was then cloned into pUC118 in E. coli. Nucleotide sequencing of the 90-bp fragment showed the presence of an open reading frame (ORF) continuing over the entire sequence. The deduced amino acid sequence of the PCR fragment was in perfect agreement with the NH₂-terminal amino acid sequence determined from the purified low specificity d-TA from Arthrobacter sp. strain DK-38 (16). We then directly performed colony hybridization with the 90-bp fragment as a probe against the established genomic library of Arthrobacter sp. strain DK-38; three positive recombinant E. coli clones were obtained from about 7,000 transformants. One of the clones showing d-TA activity (0.1 units/mg) was chosen for further characterization.

**Sequence Analysis of the dtaAS Gene**—The plasmid, pUDTA, extracted from the positive clone containing an approximately 7.2-kb insert, was directly used as the template for sequencing the dtaAS gene by the gene-walking method; the initial sequencing primer was designed based on the nucleotide sequence of the 90-bp PCR product.

**Sequence analysis of the double strand DNA revealed that the ORF consists of 1,140 bp starting with an initiation codon, ATG, and ending with a termination codon, TGA (Fig. 1). A probable ribosome-binding sequence, GGAG, is present eight bases upstream of the putative translation codon (26). The 379-residue enzyme as deduced from the DNA sequence has a molecular mass of 40,035 Da and composition as follows: Ala₂₂₀–Cys₂–Asp₂₉–Glu₂₉–Phe₁₅₄–His₁₁₀–Ile₁₁₇–Lys₁₇–Leu₉–Met₅–Asn₉–Pro₂₃–Gln₂₀–Arg₂₅–Ser₁₁–Thr₁₄–Val₁₅–Trp₇–Tyr₇. The NH₂-terminal amino acid sequence coincided with that of the purified enzyme determined by Edman degradation (Fig. 1).

**Sequence Homology with Other Proteins**—The predicted amino acid sequence showed no significant similarity to those of the currently known pyridoxal enzymes. However, in a search of protein amino acid and nucleotide sequence data bases (GenBank, EMBL, DDBJ, and Protein Data Bank) by means of the sequence similarity searching programs Blast (19) and Fasta (20), d-serine deaminase (GenBank, U41162) of Burkholderia cepacia and a hypothetical protein (GenBank, U73935) of Shewanella sp. strain SCRC-2738 were found to be
significantly similar in primary structure to low specificity D-TA. It should be mentioned that the property of the D-serine deaminase from *B. cepacia* was not reported, nor had this protein any similarity in primary structure with those of the extensively studied D-serine deaminase from *E. coli* (27) and a probable D-serine deaminase of *Bacillus subtilis* (GenBankTM, D84432), although the *E. coli* and *B. subtilis* proteins had as much as 55% identity and 69% similarity to each other. The hypothetical protein of *Shewanella* is encoded by an unnoted ORF, approximately 500 bp downstream of the eicosapentaenoic acid synthesis gene cluster of *Shewanella* sp. strain SCRC-2738 (28). The amino acid sequence alignment of D-serine deaminase and the hypothetical protein with that of low specificity D-TA is depicted in Fig. 2. D-Serine deaminase of *B. cepacia* and the hypothetical protein of *Shewanella* had 24 and 22% identities and 41 and 39% similarities to those of low specificity D-TA, respectively. Remarkably, Lys 59 of low specificity D-TA was completely conserved in the three proteins (Fig. 2).

Overexpression of the dtaAS Gene in *E. coli* Cells—The whole dtaAS gene amplified by PCR directly from *Arthrobacter* chromosomal DNA, with a putative Shine-Dalgarno sequence (GGAG) and an initiation codon (ATG), was inserted into the EcoRI site of pKK223-3. The resultant plasmid pKDTA was introduced into *E. coli* XL1-Blue MRF* cells. The nucleotide sequence of the whole amplified gene was further confirmed to have undergone no error matching during the PCR by sequencing of the double strands. The recombinant cells produced a large amount of low specificity D-TA, and the specific activity of the crude extract of *E. coli* XL1-Blue harboring pKDTA was elevated to 1.8 units/mg, which is about 180-fold over that of *Arthrobacter* sp. DK-38 (see Ref. 16, Fig. 3, and Table I). The protein was only produced in the presence of IPTG (data not shown), indicating that the tac promoter is essential for the overexpression.

Enzyme Purification—The recombinant low specificity d-TA from *Arthrobacter* sp. strain DK-38 was purified by ammonium sulfate fractionation, Butyl-Toyopearl, DEAE-Toyopearl, and Gigapite chromatography steps (Table I). About 100 mg of purified enzyme was obtained from 51 g wet cells. The purified enzyme showed a single protein band on SDS-polyacrylamide gel electrophoresis with a molecular mass of about 40 kDa (Fig. 3), the same as the value calculated from the deduced amino acid sequence of the enzyme.

PLP Requirement—The recombinant enzyme exhibited absorption maxima at 278 and 417 nm, with an A278/A417 ratio of 4.8 (curve 1, Fig. 4). The solution of the pure enzyme was distinctly yellow. As has been demonstrated with other PLP-containing enzymes, the absorption peak around 417 nm is characteristic of an azomethine linkage between the coenzyme and a protein amino group. Reduction of the enzyme with sodium borohydride by the method of Matsuo and Greenberg (29) resulted in a loss of the enzyme activity, with a disappearance of the absorption maximum at 417 nm and a concomitant increase in the A330 (data not shown). The reduced enzyme was catalytically inert, and the addition of PLP did not restore the enzyme activity. This result suggests that sodium borohydride reduces the aldimine linkage of the internal Schiff base. The holoenzyme was converted to the apoenzyme (curve 3, Fig. 4) by treatment with 1 mM hydroxylamine at 4 °C for 30 min and then dialyzed against 20 mM potassium phosphate buffer (pH 7.4). The constructed apoenzyme did not show D-TA activity.

**Fig. 1.** Nucleotide and deduced amino acid sequences of the dtaAS gene and its flanking regions. The asterisk denotes a translational stop codon. A putative Shine-Dalgarno sequence is indicated as SD with a double line. The thinly underlined amino acid sequence is identical to that determined for the purified enzyme by Edman degradation, and the boldly underlined amino acid sequence is identical to that determined for the NaBH₄ modified pyridoxyl peptide, except for Lys⁵⁹ (see text for details).
However, the activity was restored to 78% that of the native enzyme with a corresponding recovery of the $A_{417}$ (curve 2, Fig. 4) on dialysis against 20 mM potassium phosphate buffer (pH 7.4) supplemented with 10 mM PLP. In contrast, the two analogs of PLP, pyridoxal and pyridoxamine 5-phosphate, neither restored the enzyme activity nor the $A_{417}$ (data not shown). Resolution of low specificity D-TA was also carried out by treatment of the enzyme with cysteine. As shown in Fig. 4, inset, cysteine caused the disappearance of the 417-nm peak with a concomitant appearance of a peak at 330 nm (curves 2–5, Fig. 4, inset). The new absorbance peak at 330 nm disappeared on subsequent dialysis. This result indicates that cysteine resolved the enzyme by combining with the enzyme-bound PLP (417-nm peak) to form the more stable thiazolidine compound (330-nm peak) (30). All of these results show that PLP forms a Schiff base with a lysine residue of the low specificity D-TA from Arthrobacter sp. strain DK-38.

To determine the bound PLP content of low specificity D-TA, 5 mg of the enzyme was extensively dialyzed against 20 mM potassium phosphate buffer (pH 7.4) containing 10 mM PLP; the PLP concentrations inside and outside the dialysis bag were subsequently determined according to the method of Wada and Snell (25), and the difference between the inside and outside was taken as the PLP content of the enzyme. The PLP content of the enzyme was determined in triplicate to be 0.85, 0.88, and 0.94 mol/mol of 40-kDa subunits, respectively, suggesting that the enzyme has the capacity to bind 1 mol of PLP as a cofactor per mol of 40-kDa subunits.

**Identification of the PLP-binding Site**—The enzyme was treated with NaBH₄ as described under “Experimental Procedures,” and the isolation of the modified pyridoxyl peptide is depicted in Fig. 5. The amino acid sequence of the isolated peptide, which showed the absorbance peak at 330 nm, was determined by the Edman degradation procedure with a model PPSQ-10 protein sequencer. The 13 steps of degradation, except the 10th step, gave an identical amino acid sequence 50HDVALRPHAHAKH52 of the amino acid sequence deduced from the dtaAS gene (Fig. 1). The 10th step, which did not show an identifiable peak on the sequencer, was presumably the putative active site lysine residue with an asterisk.
Low Specificity D-Threonine Aldolase

Low specificity D-Threonine Aldolase

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Low specificity D-TA from Arthrobacter sp. strain DK-38 (16). Kinetic analysis further showed that the gel-filtered enzyme had the same $V_{\max}$ and $K_m$ values toward $\beta$-threo- and $\alpha$-erythro-phenylserine as those of the metal-free ones treated with EDTA, supporting the finding that the purified enzyme contained no bound metal ions, at least no activating divalent cations. Our previous work already demonstrated that the Mn$^{2+}$ ion stimulated the enzyme to give the highest aldolase activity among all cations examined (16). Mn$^{2+}$ ion was thus selected as a target for the present study. Kinetic constants of the metal-free enzyme toward various compounds were determined in the presence or absence of Mn$^{2+}$ ion, and the results are comparatively given in Table II. The $K_m$ values of the enzyme toward all substrates examined were independent of the presence or absence of Mn$^{2+}$ ion, suggesting that the metal ion may not be involved in the substrate binding. In contrast, the $V_{\max}$ values of the enzyme were significantly increased in the presence of Mn$^{2+}$ toward all examined compounds.

Furthermore, experiments were carried out to correlate the enzyme activity and amount of bound Mn$^{2+}$ ions of the recombinant low specificity D-TA. The metal-free enzyme was dialyzed twice against 1,000 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM PLP and 100 $\mu$M MnCl$_2$ for 12 h. Following gel filtration to remove free Mn$^{2+}$ ions, the amount of bound Mn$^{2+}$ ions of the enzyme was determined by atomic absorption spectrometry to be less than 0.01 mol/mol of subunit. As a consequence of the low affinity of the aldolase for the Mn$^{2+}$ ion, the stoichiometry of Mn$^{2+}$ ion binding was thus performed by equilibrium dialysis study. As shown in Fig. 6, the maximum number of bound Mn$^{2+}$ ions was determined to be 0.92 mol/mol of subunit, suggesting that the recombinant enzyme could bind 1 mol of Mn$^{2+}$ ion per mol of subunit. The maximal enzyme activity was restored on the binding of approximately 1 mol eq of Mn$^{2+}$ (Fig. 6).

**DISCUSSION**

We have previously reported the occurrence, isolation, and catalytic properties of a novel enzyme, D-threonine aldolase, that catalyzes the interconversion of D-threonine/D-allo-threonine and glycine plus acetaldehyde from Arthrobacter sp. strain DK-38 (16). To study the structural and functional relationships of the enzyme, we here cloned the dtaAS gene encoding the enzyme from the genomic DNA of Arthrobacter sp. strain DK-38 and expressed the gene in E. coli cells. Our data showed that the recombinant low specificity D-TA from Arthrobacter sp. strain DK-38, with a unique primary structure, is a novel metal-activated pyridoxal enzyme.

The recombinant low specificity D-TA from Arthrobacter sp. strain DK-38 was concluded to be a metal-activated enzyme, because of the following: 1) the purified enzyme contained no detectable metal ions; 2) the enzyme activity was stimulated by exogenous metal ions (Fig. 6, Table II, and Ref. 16); and 3) stoichiometric analysis revealed that the enzyme could bind 1 mol of Mn$^{2+}$/mol of subunit and the saturation of the metal-binding showed the maximal aldolase activity (Fig. 6). We now have insufficient data to illustrate exactly the binding mode of the divalent cation to the enzyme. However, according to the three general coordination schemes for the binding of enzyme, metal, and substrate, (i) substrate bridge complex (E-S-M), (ii) metal bridge complex (E-M-S) or S-E/M, and (iii) enzyme bridge (M-E-S), where E, S, and M represent enzyme, substrate, and metal, respectively (31), low specificity D-TA probably follows the mode of the enzyme bridge complex because 1) metal ion binding was independent of the presence of the sub-

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**FIG. 4.** Absorption spectra of the purified recombinant low specificity D-TA. Curve 1, holoenzyme; curve 2, reconstructed enzyme by dialysis of the apoenzyme against 20 mM potassium phosphate buffer (pH 7.4) supplemented with 10 mM PLP; curve 3, holoenzyme constructed by hydroxylamine treatment. The spectra were taken with 20 mM potassium phosphate buffer (pH 7.4) at 20 °C at a protein concentration of 1.1 mg/ml. Inset, variation in the absorption spectrum of the recombinant low specificity D-TA after the addition of cysteine. Curve 1, holoenzyme (4.3 mg/ml); curves 2–6, the absorption spectra of the enzyme (4.3 mg/ml) after addition of 100, 200, 300, 400, and 500 mM neutral cysteine, respectively. Each curve was taken 10 min after the addition of cysteine.

**FIG. 5.** Isolation of pyridoxyl peptide from trypsin-digested low specificity D-TA by reversed phase high pressure liquid chromatography. Elution of pyridoxyl peptide (downward) by absorbance at 330 nm and that of other peptides (upward) was by absorbance at 215 nm. The collected fraction showing an absorbance peak at 330 nm was further purified by repeated high pressure liquid chromatography and subjected to a protein sequencer for the amino acid sequence analysis.

cofactor-binding lysine residue. Remarkably, this lysine residue is completely conserved in the three proteins aligned as previously mentioned (Fig. 2).

Metal Requirement for the Aldolase Activity—To qualitatively analyze the bound metal ions of the purified recombinant low specificity D-TA from Arthrobacter sp. strain DK-38, gel filtration (HiLoad Superdex 200, Amersham Pharmacia Biotech, Uppsala, Sweden) was employed to remove free metal ions attached to the enzyme. The bound metal ions of the enzyme were subsequently analyzed with a mode ICP-1000III sequential plasma spectrometer (Shimadzu, Kyoto, Japan); the enzyme so treated was determined to contain no detectable metal ions, such as Mn$^{2+}$, Mg$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Fe$^{2+}$, and Ca$^{2+}$, which were previously shown to be activators of the wild-type low specificity D-TA from Arthrobacter sp. strain DK-38 (16).
Low Specificity d-Threonine Aldolase

**Table II**

Steady-state kinetic constants of the metal-free low specificity d-TA from *Arthrobacter* sp. strain DK-38 determined with and without MnCl₂

The values of the kinetic constants are given as mean ± standard deviation of three independent determinations.

| Compound          | With MnCl₂ | Without MnCl₂ | With MnCl₂ | Without MnCl₂ | With MnCl₂ | Without MnCl₂ |
|-------------------|------------|---------------|------------|---------------|------------|---------------|
|                   | V_max      | K_m           | V_max      | K_m           | V_max      | K_m           |
| d-Threonine       | units mg⁻¹ | m⁻¹ | 32.1 ± 1.3 | 3.0 ± 1.0 | 8.4 ± 0.3 | 8.8 ± 0.9 | 3.8 ± 0.3 | 0.3 |
| d-αllo-Threonine  |            |               | 34.9 ± 1.0 | 2.8 ± 1.1 | 4.4 ± 0.8 | 4.3 ± 0.8 | 7.9 ± 0.7 | 0.7 |
| dit-threo-Phenylserine | 209.8 ± 5.3 | 4.9 ± 0.8 | 5.4 ± 1.2 | 5.9 ± 1.2 | 38.9 ± 0.8 | 0.8 |
| dit-erythro-Phenylserine | 164.4 ± 1.6 | 1.9 ± 1.1 | 5.3 ± 0.5 | 4.9 ± 0.5 | 31.0 ± 0.4 | 0.4 |
| dit-threo-β-(3,4-Methylenedioxyphenylserine) | 129.2 ± 3.4 | 9.2 ± 2.1 | 1.8 ± 0.5 | 1.8 ± 1.4 | 71.8 ± 5.1 | 5.1 |
| dit-erythro-β-(3,4-Methylenedioxyphenylserine) | 16.0 ± 1.5 | 1.9 ± 1.1 | 2.5 ± 0.2 | 2.0 ± 0.4 | 6.4 ± 1.0 | 0.4 |
| dit-threo-β-(3,4-Dihydroxyphenylserine) | 84.6 ± 5.4 | 8.4 ± 1.2 | 1.2 ± 1.2 | 1.4 ± 0.6 | 70.5 ± 6.0 | 0.6 |

* The unresolved dit-threo-β-phenylserine, dit-erythro-β-phenylserine, dit-threo-β-(3,4-methylenedioxyphenylserine), and dit-erythro-β-(3,4-methylenedioxyphenylserine) were used as substrates, and the d-form stereoisomers were shown to be specifically cleaved as follows: the reaction mixture comprising 20 μmol of the d-form amino acid, 100 μmol of Hepes-NaOH buffer (pH 8.0), 0.05 μmol of PLP, 0.1 μmol of MnCl₂, and 1.2 units of the enzyme, in a final volume of 1 ml, was kept at 30 °C for 30 min. High performance liquid chromatography analysis (see "Experimental Procedures") confirmed that the d-form stereoisomers disappeared, whereas the l-form stereoisomers remained unchanged. Low specificity l-TA from *Pseudomonas* sp. NCIMB 10558 (14) was used as control to show that the l-form stereoisomers were stereospecifically cleaved, whereas d-form stereoisomers remained unchanged.

In summary, our findings reported here showed that low specificity d-TA from *Arthrobacter* sp. strain DK-38, with a unique primary structure, is a novel metal-activated pyridoxal enzyme, and Lys⁵⁹ of the enzyme was determined to be the pyridoxal binding site. To study further the catalytic activity as follows: (i) mechanisms involving the metal ion in a static structural role wherein binding activates the enzyme by simply stabilizing the catalytically active conformation of the protein and (ii) mechanisms where the metal ion plays a dynamic role in which binding selectively assists one or more of the protein conformational transitions essential for complementarity between enzyme site and the structure of an activated complex (32). It would be interesting to reveal whether divalent cations play the similar role in the low specificity d-TA catalysis.

**PLP-dependent enzymes catalyze manifold reactions in the metabolism of amino acids.** On the basis of a computer analysis, Alexander et al. (33) classified most of the known PLP-dependent enzymes into α, β, and γ families correlating in most cases with their regio-specificity. The α enzymes, with a few exceptions, catalyze the transformation of amino acids in which the covalency changes are limited to the α-carbon atom that carries the amino group forming the aldimine linkage to the coenzyme, such as serine hydroxymethyltransferase, 5-aminolevulinate synthase, and 8-amino-7-oxononanoate synthase. The β and γ enzymes catalyze β-replacement or β-elimination and γ-replacement or γ-elimination reactions, respectively. We have recently cloned and determined the primary structures of several l-type threonine aldolases, l-αllo-TA from *A. jandaei* DK-39 (15) and three low specificity l-TAs from *S. cerevisiae* S288C (13), *Pseudomonas* sp. strain NCIMB 10558 (14), and *E. coli* GS245.² These four l-type TAs with a significant amino acid sequence identity to one another showed no structural similarity to other PLP-dependent enzymes (14), although they belong reaction-specifically to the α family. In a search of protein sequence data bases (GenBank™, EMBL, DDBJ, and PDB) using either the total or partial sequence containing Lys³⁵ as a central amino acid as a probe, low specificity d-TA from *Arthrobacter* sp. strain DK-38 showed neither similarity in primary structure to the members belonging to the α, β, and γ families nor to the four l-type TAs, suggesting that the low specificity d-TA probably represents another new family of pyridoxal enzyme.

² J.-Q. Liu, T. Dairii, N. Itoh, M. Kataoka, S. Shimizu, and H. Yamada, submitted for publication.
role of Lys\textsuperscript{59} and the activation mechanism of the divalent cation, site-directed mutagenesis experiments and spectroscopic studies are on the way.

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MOLECULAR CLONING AND COFACTOR CHARACTERIZATION

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