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Characterization of a thermostable 2,4-diaminopentanoate dehydrogenase from *Fervidobacterium nodosum* Rt17-B1

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2,4-Diaminopentanoate dehydrogenase (2,4-DAPDH), which is involved in the oxidative ornithine degradation pathway, catalyzes the NAD$^+$- or NADP$^+$-dependent oxidative deamination of (2*R*,4*S*)-2,4-diaminopentanoate (2,4-DAP) to form 2-amino-4-oxopentanoate. A *Fervidobacterium nodosum* Rt17-B1 gene, *Fnod_1646*, which codes for a protein with sequence similarity to 2,4-DAPDH discovered in metagenomic DNA, was cloned and overexpressed in *Escherichia coli*, and the gene product was purified and characterized. The purified protein catalyzed the reduction of NAD$^+$ and NADP$^+$ in the presence of 2,4-DAP, indicating that the protein is a 2,4-DAPDH. The optimal pH and temperature were 9.5 and 85°C, respectively, and the half-denaturation time at 90°C was 38 min. Therefore, the 2,4-DAPDH from *F. nodosum* Rt17-B1 is an NAD(P)$^+$-dependent thermophilic-alkaline amino acid dehydrogenase. This is the first thermophilic 2,4-DAPDH reported, and it is expected to be useful for structural and functional analyses of 2,4-DAPDH and for the enzymatic production of chiral amine compounds. Activity of 2,4-DAPDH from *F. nodosum* Rt17-B1 was suppressed by 2,4-DAP via uncompetitive substrate inhibition. In contrast, the enzyme showed typical Michaelis–Menten kinetics toward 2,5-diaminohexanoate. The enzyme was uncompetitively inhibited by D-ornithine with an apparent K_i value of 0.1 mM. These results suggest a regulatory role for this enzyme in the oxidative ornithine degradation pathway.

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[**Key words:** 2,4-Diaminopentanoate dehydrogenase; *Fervidobacterium nodosum*; Amino acid dehydrogenase; Ornithine metabolism; Deamination; Amination; Thermophilic enzyme]

2,4-Diaminopentanoate dehydrogenase (2,4-DAPDH, EC 1.4.1.12) catalyzes NAD$^+$- or NADP$^+$-dependent oxidative deamination of (2*R*,4*S*)-2,4-diaminopentanoate (2,4-DAP) at carbon 4 to form 2-amino-4-oxopentanoate (AKP) (1). In the 1970s, this enzyme was first discovered in a crude extract of *Clostridium sticklandii* as a part of the oxidative ornithine degradation pathway (Fig. 1) (2–4), and the genes implicated in this pathway were recently identified through a metagenomics approach in an anaerobic digester from a wastewater treatment plant (5). The oxidative ornithine degradation pathway has also been verified in several anaerobic genera, including *Clostridium*, *Thermoanaerobacter*, *Propionibacterium*, and *Fervidobacterium*. The first step in this pathway is the conversion of L-ornithine to the D isomer by ornithine racemase (6). D-Ornithine is then converted to 2,4-DAP by D-ornithine aminomutase (OAM), an adenosylcobalamine (AdoCbl) and pyridoxal phosphate (PLP)-dependent enzyme, in which the amino group at carbon 5 is migrated to carbon 4 (7). 2,4-DAP is then oxidatively deaminated to form AKP. In the final step in this pathway, AKP undergoes a thiolytic cleavage, which is catalyzed by AKP thiolase with coenzyme A, to form acetyl-CoA and D-alanine (8).

Chiral amines are important starting materials for the synthesis of pharmaceuticals and agrochemicals. To obtain these chiral amine compounds, a variety of chemical and enzymatic methods have been utilized. Some examples include the enzymatic synthesis of chiral amines with lipases (9,10) and ω-amino acid aminotransferases (11,12). One drawback of most of these strategies is that they require auxiliary compounds and involve multistep transformations. In addition, in synthesis methods using an aminotransferase, the yield of the product is often unsatisfactory due to the reaction equilibrium. These drawbacks may be overcome by using amino acid dehydrogenases. Amino acid dehydrogenases catalyze the reduction of α-keto acids with concomitant amination of the substrates with NAD(P)H and an ammonium ion. Therefore, these enzymes are useful for producing chiral amines from the corresponding α-keto acid compounds in one step along with an established NAD(P)H recycling system. The product yield might also be enhanced by increasing the concentration of NAD(P)H with an NAD(P)H regeneration system. However, despite these advantages, the applications for amino acid dehydrogenases have been limited to the production of α-amino acids. 2,4-DAPDH may expand the application range of amino acid dehydrogenases for the production of other chiral amine

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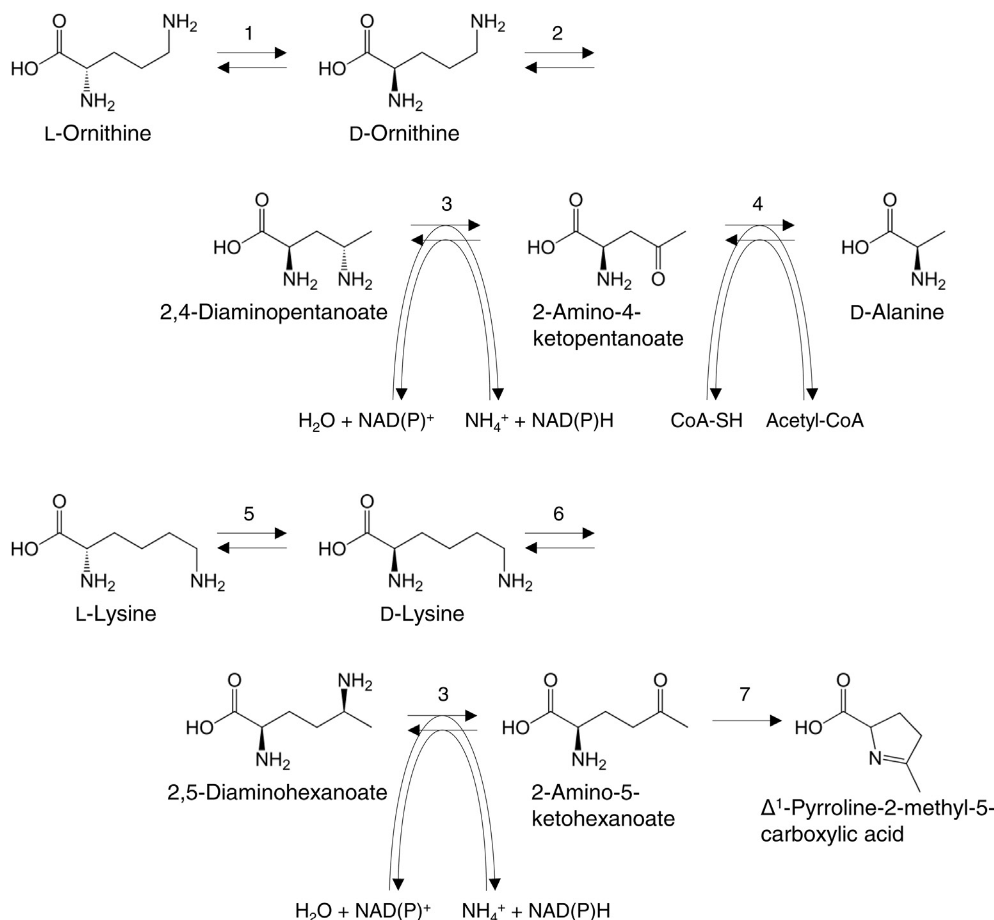

FIG. 1. The ornithine oxidative degradation pathway and putative lysine degradation pathway. Ornithine racemase, D-ornithine 4,5-aminomutase, 2,4-diaminopentanoate dehydrogenase, 2-amino-4-ketopentanoate thiolase, lysine racemase, and D-lysine 5,6-aminomutase catalyze reactions 1, 2, 3, 4, 5, and 6, respectively. Reaction 7 is non-enzymatic intramolecular cyclization.

compounds, because 2,4-DAPDH should catalyze the amination of the carbonyl group at the γ position.

Thermostable enzymes from thermophilic organisms have been used extensively in industry because these enzymes are inherently stable in harsh industrial processes. *Fervidobacterium* belongs to the eubacterial order of *Thermotogales*, which includes the most extremely thermophilic eubacteria presently known. It can grow at temperatures above 60°C with an optimal temperature of approximately 80°C (13). In this study, we carried out gene cloning, overexpression, purification, and biochemical characterization of a thermostable 2,4-DAPDH from the thermophilic anaerobic bacterium, *Fervidobacterium nodosum* Rt17-B1. This is the first report of a thermophilic 2,4-DAPDH. The role of this enzyme in the oxidative ornithine degradation pathway is also discussed.

MATERIALS AND METHODS

Materials Restriction enzymes and kits for genetic manipulation were obtained from Takara Bio (Kyoto, Japan), New England Biolabs (Ipswich, MA, USA), and Stratagene (La Jolla, CA, USA). The pET14b expression vector was purchased from Novagen (Madison, WI, USA). His-bind Resin was obtained from Novagen. All other reagents were of analytical grade and were from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan).

Cloning of the 2,4-DAPDH gene from *F. nodosum* Rt17-B1 Genomic DNA was isolated from *F. nodosum* Rt17-B1 (DSMZ, Braunschweig, Germany) using a DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. The gene encoding 2,4-DAPDH was amplified by overlap extension PCR to remove the intrinsic NdeI site in the coding region. Two separate amplification reactions were performed using Phusion DNA polymerase (Finnzymes, Espoo, Finland), 100 ng of genomic DNA as a template, and the following two sets of primers: Fnod_1646 NdeI N (5′-GCGGGAATTCCATATGCGTATAGTTACTTGGGG-3′) and Fnod_1646 deNdeI R (5′-CATTTATCGCCAACGTATGCTTTTGCTG-3′) to amplify the DNA coding for the N-terminal part of the protein, and Fnod_1646 deNdeI F (5′-CAGCAAAAGCATACGTTGGCGATAAATG-3′) and Fnod_1646 BamHI C (5′-GCCGCGGATCCTCATTCCATTTGAGAAAGGATTG-3′) to amplify the DNA coding for the C-terminal part of the protein. The underlined sequences indicate the restriction sites for NdeI and BamHI, respectively. The double-underlined sequences indicate the sites that anneal to the intrinsic NdeI site in the coding region. The PCR products were mixed and used as templates in a second PCR to amplify the full-length gene using Fnod_1646 NdeI N and Fnod_1646 BamHI C. The amplified product was digested with NdeI and BamHI and inserted into the corresponding sites of pET14b to generate an N-terminal His6-tagged protein. The recombinant plasmid was designated pET_Fnod_DAPDH.

Expression and purification of 2,4-DAPDH pET_Fnod_DAPDH was introduced into *Escherichia coli* Rosetta (DE3), and the cells were grown in LB medium containing 0.1 mM IPTG at 28°C for 15–17 h. The cells were harvested by centrifugation, resuspended in 50 mM Tris–HCl (pH 8.0), and homogenized by sonication. The homogenate was centrifuged at 25,000 ×*g* for 40 min at 4°C. The supernatant was loaded onto a His-bind column (10 mL) equilibrated with 50 mM Tris–HCl (pH 8.0). The enzyme was eluted with a 600-mL linear gradient of 0–500 mM imidazole in the same buffer. The enzyme fractions were pooled and dialyzed against 50 mM Tris–HCl (pH 8.0). The final preparation of the enzyme was stored at −80°C until use.

Synthesis of 2,4-DAP 2,4-DAP was obtained from D-ornithine by enzymatic synthesis using OAM. To prepare OAM, which is comprised of two subunits, OraS and OraE, the genes coding for these subunits were amplified by PCR from *F. nodosum* genomic DNA using the following two sets of primers: FnodOAMS_N_Bam (5′-GGAGGGGATCCGATGAAACCAAGGCCG-3′) and FnodOAMS_C_Hind (5′-TTTTCTTGGGTCGAGTTAAGCTTTTATTTAC-3′) for *oraS* and FnodOAME_N_Nde (5′-GCGGTGAGTAACATATGGACAAAC-3′) and FnodOAME_C_Xho (5′-GAATTTGACTCGAGTTATTTTTGAGATTC-3′) for *oraE*. The underlined sequences indicate the restriction sites for BamHI, HindIII, NdeI, and XhoI, respectively. The *oraS* PCR product was digested with BamHI and HindIII and inserted into the corresponding

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