J. Biochem. **143,** 603–609 (2008)
doi:10.1093/jb/mvn010

Gene Identification and Characterization of the Pyridoxine Degradative Enzyme 4-Pyridoxic Acid Dehydrogenase from the Nitrogen-fixing Symbiotic Bacterium *Mesorhizobium loti* MAFF303099†

Fei Ge[1], Nana Yokochi[1], Yu Yoshikane[1], Kouhei Ohnishi[2] and Toshiharu Yagi[1,*]

[1]Department of Bioresources Science, Faculty of Agriculture, Kochi University, Nankoku, Kochi 783-8502; and [2]Research Institute of Molecular Genetics, Kochi University, Nankoku, Kochi 783-8502, Japan

Received December 8, 2007; accepted January 12, 2008; published online January 23, 2008

The gene encoding 4-pyridoxic acid dehydrogenase was identified as mlr6792 in a chromosome of a nitrogen-fixing symbiotic bacterium *Mesorhizobium loti* MAFF303099. The enzyme is the fourth enzyme in the vitamin B_6 (pyridoxine)-degradation pathway I. The recombinant enzyme with a his-tag over-expressed in *Escherichia coli* cells was a membrane-bound protein, and purified to homogeneity. The enzyme was a monomeric protein with a molecular weight of 59,000, and a flavoprotein containing one mole of FAD per mole of subunit. The optimum pH and temperature, and K_m for 4-pyridoxic acid were pH 8.5 and 30°C, and 29 μM, respectively. The enzyme was a glucose-methanol-choline (GMC) family protein with two signature patterns, FAD-binding residues, a putative active site histidine residue and a probable transmembrane segment.

Key words: membrane-bound enzyme, 4-pyridoxic acid dehydrogenase, pyridoxine-degradation, *Mesorhizobium loti*, vitamin B_6.

Abbreviations: KP buffer, potassium phosphate buffer; DCIP, 2, 6-dichloroindophenol; GMC, glucose-methanol-choline; FHMPC, 5-formyl-3-hydroxy-2-methylpyridine-4-carboxylic acid; HMPDC, 3-hydroxy-2-methylpyridine-4, 5-dicarboxylic acid; HMPC, 3-hydroxy-2-methylpyridine-5-carboxylic acid.

Two different but related degradation pathways of free forms of vitamin B_6 (pyridoxine, pyridoxal and pyridoxamine) have been found in bacteria that can use pyridoxine as a sole source of carbon and nitrogen (*1*). We have found that *Mesorhizobium loti*, a nitrogen-fixing symbiotic bacterium, possesses the degradation pathway I (Fig. 1) for pyridoxine (*2*), and identified seven genes and two candidate ones out of total nine genes (*3*). The genes encoding pyridoxine 4-oxidase (*2*), pyridoxal-4-dehydrogenase (*4*), 4-pyridoxolactonase (*5*), 4-pyridoxic acid dehydrogenase (*6*), 3-hydroxy-2-methyl-pyridine-4,5-dicarboxylic acid decarboxylase (*7*) and 3-hydroxy-2-methyl-pyridine-5-carboxylic acid oxygenase (*8*), which are the first, second, third, fourth, fifth and seventh enzyme in the degradation pathway for pyridoxine, were over-expressed, and the recombinant enzymes were characterized by us, and Begley and co-workers. The recombinant pyridoxamine-pyruvate aminotransferase which is used for degradation of pyridoxamine was also characterized (*9*, *10*).

4-Pyridoxic acid dehydrogenase (EC 1.1.99.−) is the fourth enzyme, and catalyses conversion of 4-pyridoxic acid to 5-formyl-3-hydroxy-2-methylpyridine-4-carboxylic acid (Fig. 1). Although we have identified mlr6792 gene as a candidate for encoding 4-pyridoxic acid dehydrogenase (*3*), the recent study reported that mlr6793 gene rather than mlr6792 gene encodes the enzyme (*6*). However, in contrast to the previous report (*11*), which had reported that 4-pyridoxic acid dehydrogenase from *Pseudomonas* MA-1 was a membrane-bound enzyme with a subunit molecular weight of 63,000 and 61,000, the recombinant enzyme encoded by mlr6793 gene was a soluble enzyme with a subunit molecular weight of 33,000, and showed only about 700-fold lower k_{cat} value ($0.01\,s^{-1}$) than that ($7.3\,s^{-1}$) of the membrane-bound enzyme (*6*).

Here, we have cloned mlr6792 gene, expressed it in *Escherichia coli* B834(DE3) cells, and characterized the recombinant enzyme. It was a membrane-bound protein with a subunit molecular weight of 59,000 and showed a specific activity similar to that of the *Pseudomonas* enzyme (*11*). The results showed that mlr6792 gene indeed encodes the membrane-bound 4-pyridoxic acid dehydrogenase. Thus, the primary structure of the membrane-bound 4-pyridoxic acid dehydrogenase was elucidated for the first time here.

MATERIALS AND METHODS

Preparation of Membrane Fraction of M. loti—*Mesorhizobium loti* cells (1 g) grown in PN synthetic medium, which contains pyridoxine as a sole source of carbon and nitrogen as described previously (*8*), were suspended in 5 ml of 50 mM potassium phosphate buffer

*To whom correspondence should be addressed. Tel: +81 88 864 5191, Fax: +81 88 864 5191, E-mail: yagito@kochi-u.ac.jp
†The DDBJ Accession No of 4-pyridoxic acid dehydrogenase is AB 362509.

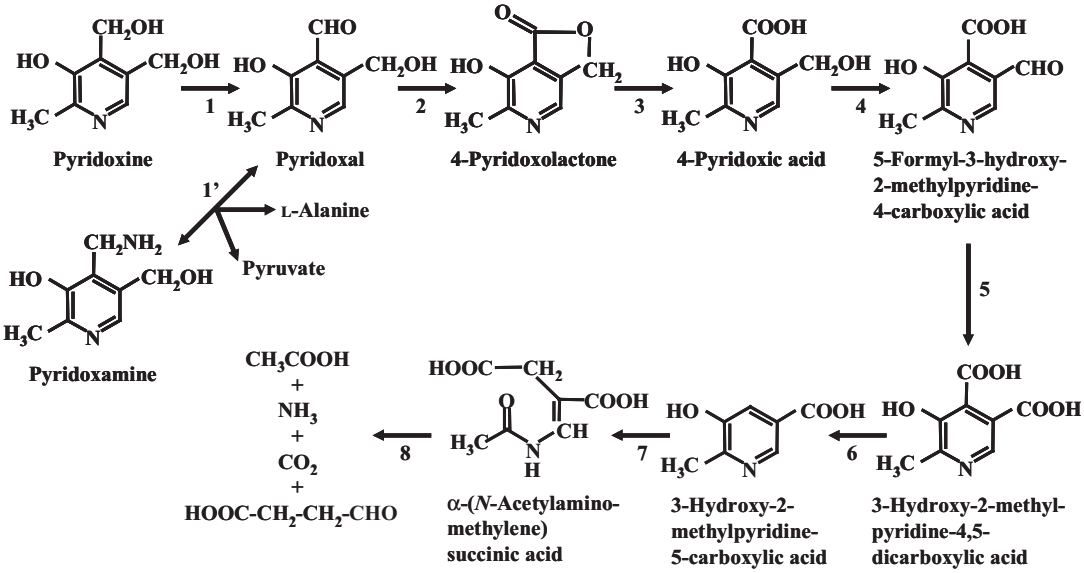

Fig. 1. **Degradation pathway I for vitamin B_6.** The degradation pathway I for vitamin B_6 consists of nine enzymes steps: *1*, pyridoxine 4-oxidase; *1′*, pyridoxamine-pyruvate aminotransferase; *2*, pyridoxal 4-dehydrogenase; *3*, 4-pyridoxolactonase; *4*, 4-pyridoxic acid dehydrogenase; *5*, 5-formyl-3-hydroxy-2-methylpyridine-4-carboxylic acid (FHMPC) dehydrogenase; *6*, 3-hydroxy-2-methylpyridine-4, 5-dicarboxylic acid (HMPDC) decarboxylase; *7*, 3-hydroxy-2-methylpyridine-5-carboxylic acid (HMPC) dioxygenase; *8*, α-(*N*-acetylaminomethylene) succinic acid amidohydrolase.

(KP buffer, pH 8.0). The suspension was sonicated thoroughly, and then subjected to centrifugation at 10,000*g* for 20 min at 4°C to prepare a crude extract. Then, the crude extract was ultracentrifuged at 100,000*g* for 90 min at 4°C. The precipitate was a membrane fraction. It was suspended in 0.2 ml of 50 mM KP buffer (pH 8.0).

Cloning and Expression of the Gene Encoding 4-Pyridoxic Acid Dehydrogenase—The chromosomal DNA of *M. loti* MAFF303099, prepared as described previously (*2*), was used as the template for PCR. The forward primer was 5′-CATATGCCGCCGCACGCGGAAAGTTA CGACTAT-3′ with a *Nde*I site (underlined). The reverse primer was 5′-GAATTCTCAgtggtggtggtggtggtgTTGCA CCTGCCCTGCCTTCTTTTG-3′ with an *Eco*RI site (underlined) and 6×His-tag (lower case). The PCR conditions were according to KOD -Plus- protocol (TOYOBO). The amplified DNA fragment was then inserted into a pTA2 vector according to the TArget Clone -Plus- protocol (TOYOBO), and then the sequence of the cloned gene in the constructed plasmid (pTA2-6792-histag) was confirmed by ABI PRISM 31000-Avant Genetic Analyzer (Applied Biosystems). Plasmid pTA2-6792-histag was digested with *Nde*I and *Eco*RI, and then the *Nde*I/*Eco*RI fragment was inserted into the *Nde*I/*Eco*RI sites of pET-21a (TaKaRa) to obtain an expression plasmid pET21a-6792-histag. *Eschrichia coli* B834(DE3) cells (Novagen) were transformed with pET21a-6792-histag. The transformant cells were cultivated in a LB medium containing 50 μM ampicillin at 23°C for purification of the enzyme.

Assay for 4-Pyridoxic Acid Dehydrogenase and Protein—The oxidation of 4-pyridoxic acid was measured at 25°C by monitoring the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm in the presence of phenazine methosulphate. A reaction mixture (1 ml) consisted of 0.1 M KP buffer, pH 8.0, 2.5 mM 4-pyridoxic acid, 48 μM DCIP, 0.1 mM phenazine methosulphate, and the enzyme. The reaction was initiated by addition of 4-pyridoxic acid. One unit of enzyme activity was defined as the amount that catalysed the reduction of 1 μmol of DCIP per minute. A molar extinction coefficient for DCIP of 21,000 $M^{-1}cm^{-1}$ at 600 nm (*11*) was used for activity calculations.

The protein concentration was determined by the dye-binding method with BSA as the standard (*12*).

Purification of 4-Pyridoxic Acid Dehydrogenase—Harvested recombinant *E. coli* B834 (DE3)/pET21a-6792-histag cells (1 g) were suspended in 10 ml of 50 mM KP buffer (pH 8.0). The suspension was sonicated thoroughly, and then subjected to centrifugation at 10,000*g* for 20 min at 4°C. The supernatant (10 ml) obtained was the crude extract, which contained membrane fragments and soluble proteins. Then, the crude extract was ultracentrifuged at 100,000*g* for 90 min at 4°C. The precipitated membrane fraction was suspended in 4 ml of 20 mM HEPES-Na (pH 8.0) containing 10% glycerol, 0.5 mM DTT, 5 μM FAD and 40 mM *n*-heptyl-β-D-thioglucoside (Buffer A), and then kept at 4°C overnight to solubilize the membrane proteins. The suspension was ultracentrifuged at 100,000*g* for 60 min at 4°C again. The supernatant obtained was used as a solubilized membrane fraction. The solubilized membrane fraction (2 ml) was loaded on a Ni–NTA-affinity column (Qiagen, 1 × 2 cm) equilibrated with 20 mM HEPES-Na, pH 8.0, containing 300 mM NaCl, 0.1% TritonX-100, 5 μM FAD, 0.5 mM DTT and 10 mM imidazole (Buffer B containing 10 mM imidazole). Then, the column was consecutively washed with 10 ml (each) of Buffer B containing 20 mM, 50 mM and 100 mM imidazole.

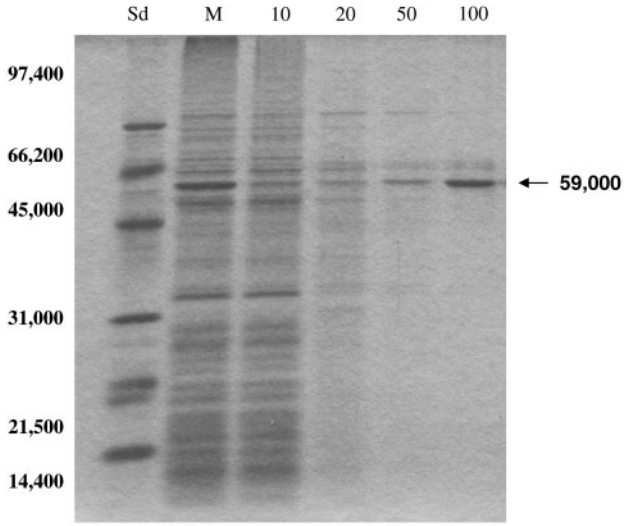

Fig. 2. **Elution pattern, homogeneity, and molecular weight of 4-pyridoxic acid dehydrogenase.** Lane Sd, the molecular mass standard markers; lane M, the solubilized membrane fraction (4.5 μg of protein); lane 10 (4.2 μg), fraction eluted with Buffer B containing 10 mM imidazole; lane 20 (1.2 μg), that with 20 mM imidazole; lane 50 (1.0 μg), that with 50 mM imidazole; lane 100 (1.0 μg), that with 100 mM imidazole.

The recombinant 4-pyridoxic acid dehydrogenase was eluted from the column with Buffer B containing 50 mM and 100 mM imidazole (Fig. 2). The fractions containing active enzyme were pooled and concentrated using VIVASPIN 6 (VIVASCIENCE Sartorius group).

Other Analytical Methods—Purity of the enzyme and a molecular weight of the subunit were estimated by SDS–PAGE as described previously (*13*). The molecular weight of the native enzyme was estimated by gel filtration (COSMOSIL 5diol-120II HPLC column, Nacalai Tesque): the column was equilibrated with Buffer A containing 0.15 M NaCl, and eluted at a flow rate of 0.5 ml/min. A calibration curve was made based on the elution volume of lactate dehydrogenase (Mr, 140,000), alanine aminotransferase (Mr, 110,000), malate dehydrogenase (Mr, 72,000) and cytochrome *c* (Mr, 12,400). The molecular weight of the native enzyme was also estimated by density gradient centrifugation (*14*). Samples (20 μl) were layered on a 3.5 ml density gradient of sucrose (5 to 20%) in Buffer B, and centrifuged at 180,000*g* in a Beckman SW55Ti rotor. Catalase (Mr, 240,000), lactate dehydrogenase, alanine aminotransferase, malate dehydrogenase and cytochrome *c* were used as marker proteins.

The content of FAD in 4-pyridoxic acid dehydrogenase was determined with a fluorometric HPLC (*15*). The enzyme solution (0.37 mg/ml) dialysed against Buffer B without FAD and NaCl was treated with 10% trichloroacetic acid to dissociate FAD from enzyme protein, and then trichloroacetic acid was removed by extraction with diethyl ether (*11*). The sample obtained was applied on a Cosmosil 5C18-MS-II column (Nacalai Tesque). FAD was detected with a JASCO FP-2025 Plus fluorescence detector (an excitation at 450 nm and an emission at 530 nm).

The reaction product (5-formyl-3-hydroxy-2-methylpyridine-4-carboxylic acid) was identified by the isocratic HPLC method with a UV (at 316 nm) detection system as described previously (*16*). Kinetic parameters were determined using curve fitting software (Kaleida Graph) to fit the Michaelis–Menten equation with the Levenberg–Marquardt algorithm.

The protein sequence of the enzyme was also determined after the digestion with an *N*-acetyl deblocking aminopeptidase (TaKaRa). The transmembrane segments in the amino acid sequence of 4-pyridoxic acid dehydrogenase was deduced by the HMMTOP server (http://enzim.hu/hmmtop/server/hmmtop.cgi) (*17*).

RESULTS AND DISCUSSION

Localization of 4-Pyridoxic Acid Dehydrogenase Activity in M. loti Cells—The crude extract from *M. loti* cells grown in the pyridoxine medium showed weak but measurable 4-pyridoxic acid dehydrogenase activity (0.01 ± 0.004 U/mg). The specific activity of the membrane fraction was 0.1 ± 0.03 U/mg. About 90% of the total activity was found in the membrane fraction. Thus, 4-pyridoxic acid dehydrogenase is membrane-bound in *M. loti* cells like in *Pseudomonas* MA-1 cells (*11*).

Cloning and Expression of mlr6792 Gene, and Purification of the Recombinant Enzyme—The *E. coli* B834(DE3) cells transformed with the expression plasmid, pET21a-6792-histag, grown at 23°C showed a high 4-pyridoxic acid dehydrogenase activity: the specific activity (0.3 ± 0.05 U/mg) in the crude extract was 30-fold higher than that of *M. loti* cells grown in the pyridoxine medium. The membrane fraction from the transformed cells showed a high specific activity (3.3 ± 0.47 U/mg). About 94% of the total activity in the transformed cells was found in the membrane fraction. Thus, 4-pyridoxic acid dehydrogenase is also membrane-bound in the transformed *E. coli* cells.

It is noted that the transformed cells showed the high 4-pyridoxic acid dehydrogenase activity only when they were cultivated at low temperature 23°C: an inactive aggregated enzyme protein was found in the cells cultivated at 37°C. Thus, it was suggested that cold stress was necessary for the correct folding of the recombinant 4-pyridoxic acid dehydrogenase in the *E. coli* cells. The cold stress has been reported as an effective way to make recombinant enzymes active soluble forms (*18*).

Thus, the recombinant 4-pyridoxic acid dehydrogenase was purified from the membrane fraction of the transformed *E. coli* cells. The enzyme was homogeneously purified by affinity chromatography with a Ni–NTA column (Table 1). The elution pattern and purity of the eluted fraction from the column are shown in Fig. 2.

Enzymatic Properties of 4-Pyridoxic Acid Dehydrogenase from M. loti—The purified enzyme showed a single protein band with a molecular weight of $59,000 \pm 1,000$ (mean and SD of two measurements) on a SDS–PAGE gel (Fig. 2). This molecular weight coincided with that (58,921) of the predicted protein encoded by the mlr6792 gene. The sucrose density centrifugation and the gel filtration of the native

enzyme gave similar molecular weights of 54,000 ± 900 and 57,000 ± 1,000, respectively. The results showed that the native recombinant 4-pyridoxic acid dehydrogenase is a monomeric protein.

The *Pseudomonas* enzyme showed one main band with molecular weight of 63,000 and minor one with 61,000 on a SDS–PAGE gel (*11*). The present results suggest that the *Pseudomonas* enzyme is also a monomeric enzyme, and the component of the minor band may be a contaminating protein or 4-pyridoxic acid dehydrogenase partially digested by some protease.

The enzyme was yellowish and showed absorption maxima near 380 nm and 455 nm, characteristic of flavoproteins. The amount of FAD in the enzyme was determined as 1.23 ± 0.04 mol/mol.

The amino-terminus of the recombinant enzyme was inferred to be blocked, as no amino acid was released by Edman geradation. When the enzyme was pre-digested with the *N*-acetyl deblocking aminopeptidase, which can cleave an amino-terminal amino acid residue with an acyl group, and then hydrolyse sequentially a peptide bond from the amino-terminus, a peptide sequence corresponding to 406–415 amino acids of the enzyme (shown by blue letters in the sequence of the enzyme in Fig. 4) was determined. The results showed that the aminopeptidase released the amino-terminus, and then digested the following peptide bonds until Pro405. It is hence certain that the recombinant enzyme was blocked with an acyl group.

Table 1. **Purification of recombinant 4-pyridoxic acid dehydrogenase.**

Fractions	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Membrane	6.0	22.2	3.7	100
Ni-NTA column	0.6	6.8	11.9	30.1

The optimum pH of 4-pyridoxic acid dehydrogenase from *M. loti* was around pH 8.5 as shown in Fig. 3 A, showing somewhat higher pH than that (pH 8.0) of the *Pseudomonas* enzyme (*11*). The optimum temperature for 4-pyridoxic acid dehydrogenase was 30–35°C (Fig. 3B). The enzyme was stable at 40°C or lower for 10 min, but its activity became 54% and 11% of the original level at 55°C and 60°C, respectively (Fig. 3C).

4-Pyridoxic acid dehydrogenase showed a typical Michaelis–Menten type kinetics when the effect of the 4-pyridoxic acid concentration on the reaction rate was examined (Fig. 3D). The K_m and k_{cat} were 29 ± 1.8 μM and 11 ± 2.5 s^{-1}, respectively. The enzyme was highly specific for 4-pyridoxic acid: it showed <0.01% of the activity of 4-pyridoxic acid towards pyridoxal, pyridoxine and 4-pyridoxic acid lactone, and simple α-hydroxyl acids, such as lactic acid and glycolic acid. The *Pseudomonas* enzyme also showed the high specificity toward 4-pyridoxic acid (*11*).

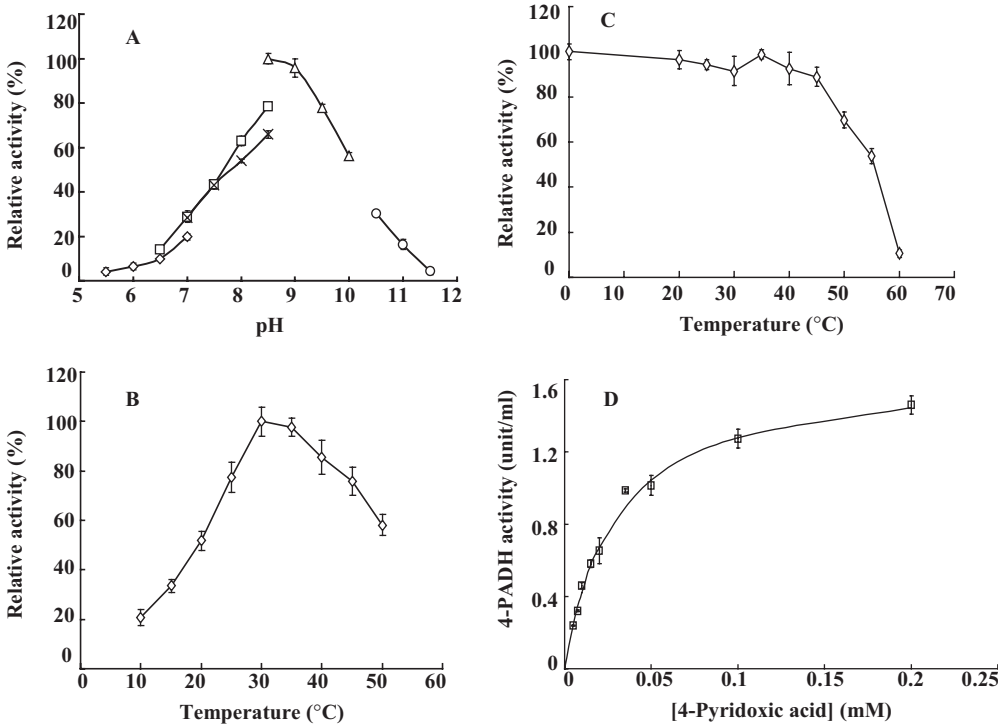

Fig. 3. **Optimum pH, optimum temperature, temperature stability and kinetics of 4-pyridoxic acid dehydrogenase.** A, optimum pH: the enzyme was assayed in 0.1 M CHES (open triangle; pH 8.5–10.0), 0.1 M MES (open diamond; pH 5.5–7.0), 0.1 M CAPS (open circle; pH 10–11), 0.1 M KP buffer (open square; pH 6.5–8.5) and 0.1 M HEPES (cross; pH 7.0–8.5). B, optimum temperature: the purified enzyme was assayed at temperatures in a range of 10–50°C. C, temperature stability: the enzyme was incubated at different temperatures for 10 min, and then cooled on ice. D, Effect of 4-pyridoxic acid concentration on the enzyme activity. The reaction was done at various 4-pyridoxic acid concentration under the standard conditions.

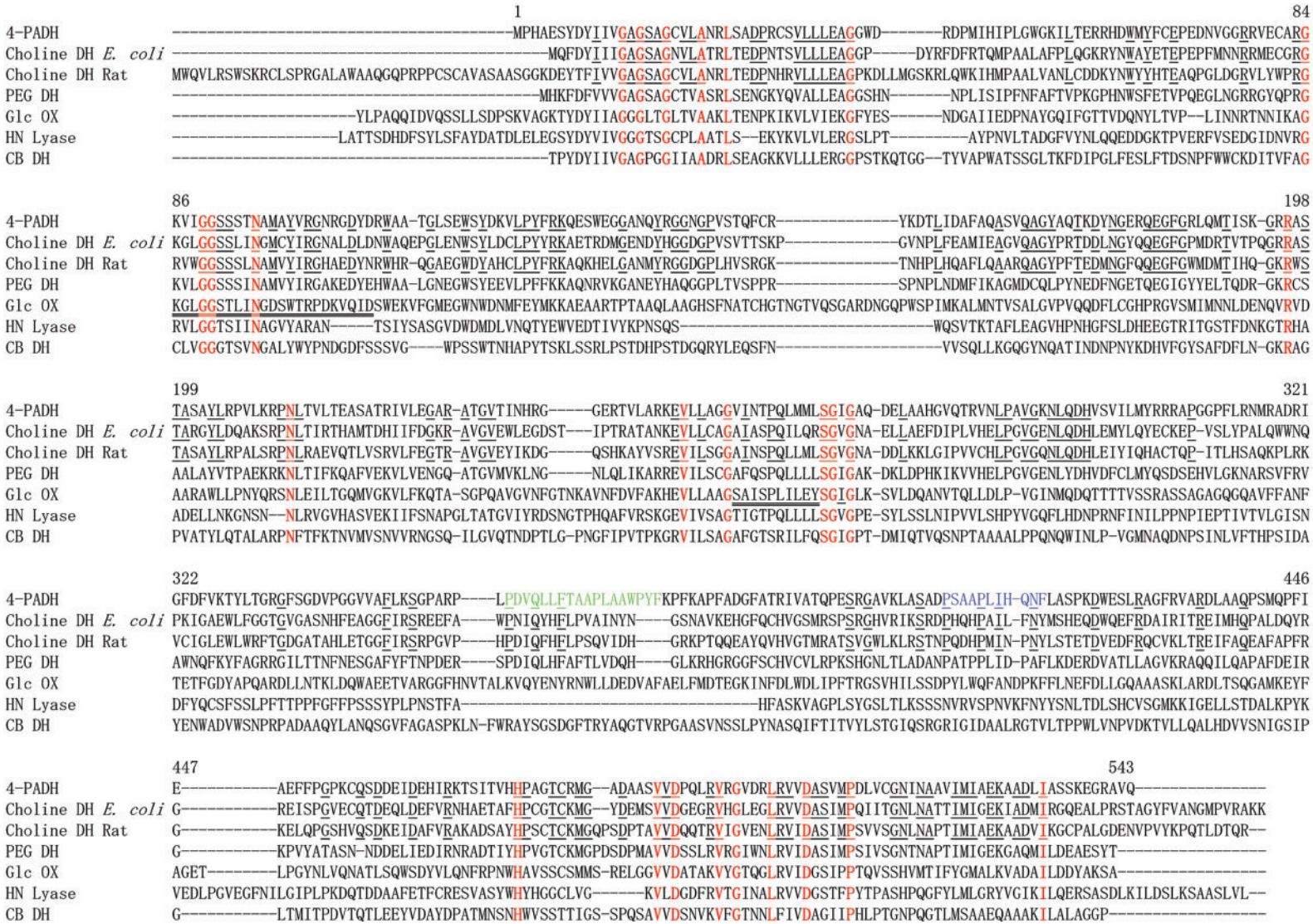

Fig. 4. **Alignment of the amino acid sequences of 4-pyridoxic acid dehydrogenase and some glucose-methanol-choline oxidoreductases.** The sequence was aligned by means of Clustal W multiple sequence alignment program. Abbreviations are: 4-PADH, 4-pyridoxic acid dehydrogenase; Choline DH *E. coli*, choline dehydrogenase from *E. coli*; Choline DH Rat, choline dehydrogenase from rat; PEG DH, polyethylene glycol dehydrogenase from *Pseudomonas* sp. PE-2; Glc OX, glucose oxidase from *P. amagasakiense*; HN Lyase, hydroxynitrile lysase from almond; and CB DH, cellobiose dehydrogenase from *Phanerochaete chrysosporium*. Residues are highlighted as follows: underlined, common to 4-pyridoxic acid dehydrogenase and choline dehydrogenases; double-underlined, glucose-methanol-choline oxidoreductase signature; red, common to all the sequences examined; blue, amino-terminal of the peptide obtained after digestion with the aminopeptidase; and green, deduced transmembrane segment.

Amino-Acid Sequence Comparisons—The amino acid sequence of 4-pyridoxic acid dehydrogenase was compared with those of other enzyme proteins by means of a BLAST search. The enzyme showed the highest (57%) identity with putative choline dehydrogenase in *Bordetella avium* 197N (CAJ49516.1, 537 residues). Other 100 of deduced GMC oxidoreductases showed 42% or higher identity. Then, six GMC oxidoreductases whose reaction and substrate specificities have been identified were selected to analyse the sequence similarities to 4-pyridoxic acid dehydrogenase. Choline dehydrogenases from *E. coli* (*19*) and rat (*20*), polyethylene glycol dehydrogenase from *Pseudomonas* sp. PE-2 (*21*), glucose oxidase from *Penicillium amagasakiense* (*22*), hydroxynitrile lyase from almond (*23*) and cellobiose dehydrogenase from *Phanerochaete chrysosporium* (*24*) showed identities of 42%, 42%, 38%, 26%, 26% and 24%, respectively. Multiple sequence alignment of these enzymes with ClustalW is shown in Fig. 4. 4-Pyridoxic acid dehydrogenase contains two GMC oxidoreductase signature sequences (PROSITE) (amino acid residues, 84–107 and 256–270): the corresponding sequences are shown by underlined letters in the sequence of the glucose oxidase. The enzyme also has amino-terminal and carboxyl-terminal consensus sequences for FAD binding. His475 is common in all of the enzymes examined. It corresponds to His516 in the glucose oxidase (*22*), which is conserved in all members of GMC oxidoreductase family and functions as a general base to abstract a proton from the carbon to be oxidized. Thus, His475 may also be an active site residue in 4-pyridoxic acid dehydrogenase. Interestingly, amino acid residues, 322–446, in 4-pyridoxic acid dehydrogenase show a very low identity (12%) with the choline dehydrogenases, and the deduced transmembrane segment (amino acid residues, 356–373, shown by green letters in Fig. 4) resides in this region. Thus, it is not contradictory to propose that 4-pyridoxic acid dehydrogenase is anchored to the plasma membrane through this segment because the choline dehydrogenases are peripheral proteins, and have no transmembrane α-helix. We are continuing our investigations into the topology of the enzyme in the bacterial cells.

We have searched the *Pseudomonas* genome database (http://www.pseudomonas.com/search.jsp) to find the *Pseudomonas* orthologue of 4-pyridoxic acid dehydrogenase. The amino acid sequence of a deduced choline dehydrogenase encoded by a gene (locus ID, PSEEN2583) in *Pseudomonas entomophila* L-48 showed the highest identity (51.93%) with that of the *M. loti* enzyme. It contained one deduced transmembrane segment, suggesting that it is membrane bound. Then, the amino acid sequences of proteins encoded by genes around PSEEN2583 were aligned with those of the other enzymes shown in Fig. 1, because they would exist as a cluster like in *M. loti* genome. The proteins encoded by PSEEN 2585, 2587, 2582 and 2579 showed identities of 43.23%, 32.6%, 29.13% and 25.99% with HMPDC decarboxylase, pyridoxal 4-dehydrogenase, 4-pyridoxolactonase and HMPC dioxygenase, respectively. However, no homologous proteins were found for the first, fifth and eighth enzymes, and pyridoxamine-pyruvate aminotransferase. Thus, the enzyme encoded by PSEEN2583 gene may be a 4-pyridoxic acid dehydrogenase but further investigations are required to exactly identify its function.

In conclusion, we have identified mlr6792 instead of mlr6793 as the membrane-bound 4-pyridoxic acid dehydrogenase-coding gene. We reported that the latter gene encodes FHMPC dehydrogenase (dismutase) which catalyses the next step of the 4-pyridoxic acid dehydrogenase reaction (Fig. 1) (*3*). We have cloned, expressed mlr6793 gene, characterized the over-expressed recombinant enzyme, and found that it indeed encodes FHMPC dehydrogenase (dismutase) (unpublished data).

REFERENCES

1. Nelson, M.J. and Snell, E.E. (1986) Enzymes of vitamin B_6 degradation. Purification and properties of 5-pyridoxic-acid oxygenase from *Arthrobacter* sp. *J. Biol. Chem.* **261**, 15115–15120
2. Yuan, B., Yoshikane, Y., Yokochi, N., Ohnishi, K., and Yagi, T. (2004) The nitrogen-fixing symbiotic bacterium *Mesorhizobium loti* has and expresses the gene encoding pyridoxine 4-oxidase involved in the degradation of vitamin B_6. *FEMS Microbiol. Lett.* **234**, 225–230
3. Yagi, T., Yokochi, N., Yoshikane Y., Yuan, B., Fumani, J., Tadokoro, M., and Ge, F. (2005) Genes encoding enzymes involved in degradation for vitamin B_6. Abstract of IUBMB Symposium, **L11R**.
4. Yokochi, N., Nishimura, S., Yoshikane, Y., Ohnishi, K., and Yagi, T. (2006) Identification of a new tetrameric pyridoxal 4-dehydrogenase as the second enzyme in the degradation pathway for pyridoxine in a nitrogen-fixing symbiotic bacterium, *Mesorhizobium loti*. *Arch. Biochem. Biophys.* **452**, 1–8
5. Funami, J., Yoshikane, Y., Kobayashi, H., Yokochi, N., Yuan, B., Iwasaki, K., Ohnishi, K., and Yagi, T. (2005) 4-Pyridoxolactonase from a symbiotic nitrogen-fixing bacterium *Mesorhizobium loti*: Cloning, expression, and characterization. *Biochim. Biophys. Acta* **1753**, 234–239
6. Mukherjee, T., Kinsland, C., and Begley, T.P. (2007) PLP catabolism: Identification of the 4-pyridoxic acid dehydrogenase gene in *Mesorhizobium loti* MAFF303099. *Bioorg. Chem.* **35**, 458–464
7. Mukherjee, T., McCulloch, K.M., Ealick, S.E., and Begley, T.P. (2007) Gene identification and structural characterization of the pyridoxal 5′-phosphate degradative protein 3-hydroxy-2-methylpyridine-4,5-dicarboxylate decarboxylase from *Mesorhizobium loti* MAFF303099. *Biochemistry* **46**, 13606–13615
8. Yuan, B., Yokochi, N., Yoshikane, Y., Ohnishi, K., and Yagi, T. (2006) Molecular cloning, identification and characterization of 2-methyl-3-hydroxypyridine-5-carboxylic-acid-dioxygenase-coding gene from the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *J. Biosci. Bioeng.* **102**, 504–510
9. Yoshikane, Y., Yokochi, N., Ohnishi, K., Hayashi, H., and Yagi, T. (2006) Molecular cloning, expression and characterization of pyridoxamine-pyruvate aminotransferase. *Biochem. J.* **396**, 499–507
10. Yoshikane, Y., Yokochi, N., Yamasaki, M., Mizutani, K., Ohnishi, K., Mikami, B., Hayashi, H., and Yagi, T. (2008) Crystal structure of pyridoxamine-pyruvate aminotransferase from *Mesorhizobium loti* MAFF303099. *J. Biol. Chem.* **283**, 1120–1127
11. Yagi, T., Kishore, G.M., and Snell, E.E. (1983) The bacterial oxidation of vitamin B_6. 4-Pyridoxic acid dehydrogenase: a

membrane-bound enzyme from *Pseudomonas* MA-1. *J. Biol. Chem.* **258**, 9419–9425
12. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Chem.* **72**, 248–254
13. Nakano, M., Morita, T., Yamamoto, T., Sano, H., Ashiuchi, M., Masui, R., Kuramitsu, S., and Yagi, T. (1999) Purification, molecular cloning, and catalytic activity of *Schizosaccharomyces pombe* pyridoxal reductase. A possible additional family in the aldo-keto reductase superfamily. *J. Biol. Chem.* **274**, 23185–23190
14. Martin, R.G. and Ames, B.N. (1961) A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J. Biol. Chem.* **236**, 1372–1379
15. Gliszczyńska-Świglo, A. and Koziolowa, A. (2000) Chromatographic determination of riboflavin and its derivatives in food. *J. Chromatogr. A* **881**, 285–297
16. Yagi, T., Matsuoka, K., and Yamamoto, S. (1993) Interaction of pyridoxal 5′-phosphate form of aspartate aminotransferase with vitamin B-6 compounds and antagonist in rabbit erythrocytes. *Biosci. Biotechnol. Biochem.* **57**, 753–759
17. Tusnady, G.E. and Simon, I. (1998) Principles governing amino acid composition of integral membrane proteins: application to topology prediction. *J. Mol. Biol.* **283**, 489–506
18. Yoshikane, Y., Yokochi, N., Ohnishi, K., and Yagi, T. (2004) Coenzyme precursor-assisted cooperative overexpression of an active pyridoxine 4-oxidase from *Microbacterium luteolum*. *Protein Expr. Purif.* **34**, 243–248
19. Lamark, T., Kaasen, I., Eshoo, M.W., Falkenberg, P., McDougall, J., and Strom, A.R. (1991) DNA sequence and analysis of the bet genes encoding the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. *Mol. Microbiol.* **5**, 1049–1064
20. Huang, S. and Lin, Q. (2003) Functional expression and processing of rat choline dehydrogenase precursor. *Biochem. Biophys. Res. Commun.* **309**, 344–350
21. Hu, X., Fukutani, A., Liu, X., Kimbara, K., and Kawai, F. (2007) Isolation of bacteria able to grow on both polyethylene glycol (PEG) and polypropylene glycol (PPG) and their PEG/PPG dehydrogenases. *Appl. Microbiol. Biotechnol.* **73**, 1407–1413
22. Wohlfahrt, G., Witt, S., Hendle, J., Schomburg, D., Kalisz, H.M., and Hecht, H.-J. (1999) 1.8 and 1.9 Å resolution structures of the *Penicillium amagasakiense* and *Aspergillus niger* glucose oxidases as a basis for modelling substrate complexes. *Acta Cryst.* **D55**, 969–977
23. Dreveny, I., Gruber, K., Glleder, A., Thompson, A., and Kratky, C. (2001) The hydroxynitrile lyase from almond: a lyase that looks like an oxidoreductase. *Structure* **9**, 803–815
24. Hallberg, B.M., Henriksson, G., Pettersson, G., Vasella, A., and Divne, C. (2003) Mechanism of the reductive half-reaction in cellobiose dehydrogenase. *J. Biol. Chem.* **278**, 7160–7166