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Gene Identification and Characterization of 5-Formyl-3-Hydroxy-2-Methylpyridine 4-Carboxylic Acid 5-Dehydrogenase, an NAD^+-Dependent Dismutase*

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A chromosomal gene, *mlr6793*, in *Mesorhizobium loti* was identified as the gene encoding 5-formyl-3-hydroxy-2-methylpyridine 4-carboxylic acid (FHMPC) dehydrogenase (dismutase) involved in the degradation pathway for pyridoxine (vitamin B_6). The homogenously purified recombinant enzyme has a molecular mass of 59.1 kDa and is a homodimeric protein. FHMPC dehydrogenase catalyses practically irreversible oxidation ($k_{cat} = 204\ s^{-1}$) of FHMPC ($K_m = 48.2\ \mu M$) by NAD^+ ($K_m = 34.3\ \mu M$) to 3-hydroxy-2-methyl-pyridine 4, 5-dicarboxylic acid (HMPDC), and practically irreversible reduction ($k_{cat} = 217\ s^{-1}$) of FHMPC ($K_m = 24.9\ \mu M$) by NADH ($K_m = 12.4\ \mu M$) to 4-pyridoxic acid. When the enzyme reaction was started with the combination of FHMPC and NAD^+ or that of FHMPC and NADH, HMPDC and 4-pyridoxic acid were produced in an almost equimolar ratio throughout the reaction. FHMPC dehydrogenase belongs to the 3-hydroxyacyl-CoA dehydrogenase family with 31% identity with the human enzyme: it has probable catalytic diad residues, *i.e.* His137 and Glu149. The H137L mutant enzyme showed no measurable activity. The E149Q one was stable in contrast to the corresponding human 3-hydroxyacyl-CoA dehydrogenase mutant, and showed unique pH optima depending on the co-substrates used for the reaction.

Key words: 5-formyl-3-hydroxy-2-methylpyridine 4-carboxylic acid (FHMPC) dehydrogenase, NAD^+-dependent dismutase, *Mesorhizobium loti*, vitamin B_6-degradation pathway.

Abbreviations: FHMPC, 5-formyl-3-hydroxy-2-methylpyridine 4-carboxylic acid; HMPDC, 3-hydroxy-2-methyl-pyridine 4, 5-dicarboxylic acid.

A nitrogen-fixing symbiotic bacterium, *Mesorhizobium loti*, possesses vitamin B_6 (pyridoxine, pyridoxamine, and pyridoxal)-degradation pathway I (*1*), in which it is converted into acetic acid, succinic semialdehyde, carbon dioxide and ammonia through nine enzyme steps (Fig. 1A) (*2*). The enzyme genes exist as a cluster on a chromosomal DNA of the bacterium (Fig. 1B). We have identified and over-expressed seven (1st, 2nd, 3rd, 4th, 7th, 8th and 1st') out of the total nine genes encoding the enzymes involved, and characterized the recombinant enzymes (*1*, *3*–*9*). The sixth gene encoding 3-hydroxy-2-methylpyridine 4, 5-dicarboxylic acid (HMPDC) decarboxylase was identified, and the tertiary structure of the enzyme was determined (*10*). Although we have assigned the *mlr6793* gene as a candidate encoding the fifth enzyme, 5-formyl-3-hydroxy-2-methylpyridine 4-carboxylic acid (FHMPC) dehydrogenase (*11*), a recent study revealed that it encodes the 4th enzyme, 4-pyridoxic acid dehydrogenase (*12*). However, we have confirmed that *mlr6792* instead of *mlr6793* encodes the 4th enzyme (*5*). Thus, the gene encoding FHMPC dehydrogenase should be definitely identified.

FHMPC dehydrogenase has been purified to homogeneity from *Pseudomonas* MA-1 and characterized (*13*). The enzyme is a homodimeric protein with a subunit molecular mass of 34 kDa, and is metal-independent. It catalyses, at almost the same reaction rate, the oxidation of FHMPC to HMPDC (Fig. 1A, reaction step 5f) with NAD^+ and the reduction of FHMPC to 4-pyridoxic acid with NADH as a hydrogen donor (Fig. 1A, reaction step 5r). The enzyme did not catalyse the oxidation of 4-pyridoxic acid with NAD^+ (*13*): this reaction (the 4th step reaction in the degradation pathway) is catalysed by a membrane-bound enzyme, 4-pyridoxic acid dehydrogenase (*5*, *14*). Thus, FHMPC dehydrogenase practically catalyses the dismutation reaction, as described previously (*13*), although it has remained to be confirmed that the enzyme cannot catalyse the reverse reduction of HMPDC with NADH.

Although many dehydrogenases catalyse the dismutation of aldehydes (*15*, *16*), as far as we know, a detailed reaction mechanism based on the tertiary structure of the enzyme protein has not been reported, and the dismutation reaction of no dehydrogenase has been

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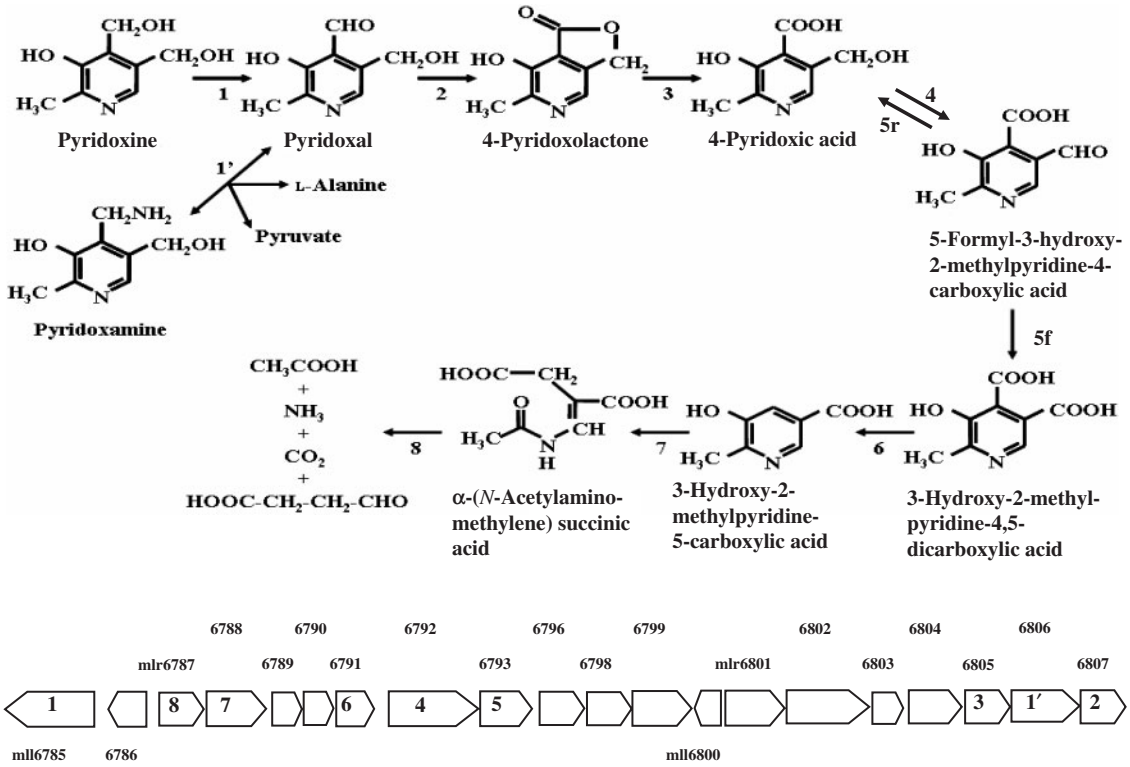


Fig. 1. **Degradation pathway I for vitamin B_6 and a cluster of genes involved in the pathway.** (A) Degradation pathway I for vitamin B_6. 1, pyridoxine 4-oxidase; 1′, pyridoxamine–pyruvate aminotransferase; 2, pyridoxal 4-dehydrogenase; 3, 4-pyridoxolactonase; 4, 4-pyridoxic acid dehydrogenase; 5, 5-formyl-3-hydroxy-2-methylpyridine-4-carboxylic acid dehydrogenase; 6, 3-hydroxy-2-methylpyridine-4,5-dicarboxylic acid decarboxylase; 7 3-hydroxy-2-methylpyridine-5-carboxylic acid dioxygenase; 8, α-(*N*-acetylaminomethylene) succinic acid amidohydrolase. (B) The cluster of genes involved in vitamin B_6 degradation in *Mesorhizobium loti* MAFF303099.

assigned to any physiological function. Horse liver alcohol dehydrogenase (EC 1.1.1.1) is among the well-known dehydrogenases, which show dismutase activity as a secondary reaction when an aldehyde is used as a substrate (*15*). This Zn-dependent enzyme has histidine and serine residues in its active site (*17*). In contrast, aldehyde dehydrogenase family enzymes, which are mostly metal-independent, contain glutamic acid and cysteine residues in their active sites (PROSITE documentation PDOC00068). Thus, determination of the amino acid residues involved in the catalytic reaction of FHMPC dehydrogenase is required for elucidation of its reaction mechanism.

Here, we have identified the *mlr6793* gene as the FHMPC dehydrogenase-encoding one, expressed it in *Escherichia coli* BL21(DE3) cells, and characterized the recombinant enzyme. The amino acid residues involved in the catalysis were determined by site-directed mutagenesis. The E149Q mutant enzyme showed characteristic properties different from those of the corresponding human 3-hydroxyacyl-CoA dehydrogenase mutant (*18*).

MATERIALS AND METHODS

Chemicals—4-Pyridoxic acid was purchased from Sigma. FHMPC was synthesized from 4-pyridoxic acid with transformed *E. coli* cells over-expressing 4-pyridoxic acid dehydrogenase (*5*) as follows. The reaction mixture (10 ml) comprising 50 mM sodium phosphate, pH 8.0, 10 mM 4-pyridoxic acid and 225 mg transformed *E. coli* cells was incubated at 30°C for 5.5 h. After the cells had been removed by centrifugation, the supernatant was applied to a Dowex 1-×8 column (2.0 × 15 cm, formate form; Dow Chemical Company). The column was washed with 200 ml of distilled water and 500 ml of 0.5 M formic acid, and then eluted with a linear gradient with 0.5–3 M formic acid (250 ml each). The collected FHMPC fraction was concentrated, and then the FHMPC obtained was re-crystallized three times with distilled water. The identification and purity of FHMPC were determined by spectrophotometric analysis (*19*) and isocratic HPLC as described below. HMPDC was purified from the culture broth of *M. loti* grown in a pyridoxine synthetic medium essentially as described previously (*19*). HMPDC was also prepared from FHMPC with the *E. coli* cells expressing FHMPC dehydrogenase described here by ion-exchange column chromatography. The purity of HMPDC was determined by spectrophotometric analysis and HPLC.

Bacterial Strains, Plasmids and Cultivation—Plasmids pNEB205A (New England BioLabs), pET21a (Novagen), pTA2 (Toyobo), pK18*mobsacB* (*20*), pKRP12 (*21*) and pBBad22K (*22*) were used. *Mesorhizobium loti* MAFF303099 was obtained from the MAFF GenBank

(Tsukuba, Japan). *Mesorhizobium loti* cells were cultured in TY medium (*1*) at 30°C. *Escherichia coli* strains JM109 and BL21(DE3) were purchased from TaKaRa Bio and Novagen, respectively. *Escherichia coli* S17-1 was obtained from the National Institute of Genetics Stock Research Center (Mishima, Japan). *Escherichia coli* cells were cultured in LB medium containing antibiotics at 37°C. The concentrations of the antibiotics used were: ampicillin, 100 μg/ml; tetracycline, 12.5 μg/ml; and kanamycin, 50 μg/ml. For preparation of a disruption mutant of *M. loti*, tetracycline, 5 μg/ml, kanamycin, 200 μg/ml and phosphomycin, 100 μg/ml, were used.

Cloning and Expression of the mlr6793 Gene—The *mlr6793* gene was amplified by PCR with the chromosomal DNA of *M. loti* MAFF303099 prepared with an AquaPure Genomic DNA Isolation Kit (Bio-Rad). PCR was performed as described previously (*3*) with primers containing deoxyuridine (dU), 5′-GGAGACAdUATGATCCGAAATATCGCCATCATCGGC-3′ (the underlining indicates an *Nde*I site) and 5′-GGGAAAGdUGCCGGCTTTGTGAGCTCGATAAA-3′. The amplified DNA fragment was ligated into the pNEB205A vector with a USER (Uracil-Specific Excision Reagent) friendly cloning kit (New England Biolabs). The constructed plasmid, pNEB6793, was introduced into and extracted from JM109 cells. After the sequence of the introduced fragment had been verified by DNA sequencing with an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems), pNEB6793 was digested with *Nde*I and *EcoR*I, and then the digested DNA fragment was inserted into the *Nde*I/*EcoR*I sites of pET21a. An *EcoR*I site existed in the pNEB205A vector. The constructed plasmid, designated as pET6793, was introduced into BL21(DE3) cells.

The transformed cells were aerobically grown in 5 ml of LB medium containing ampicillin at 37°C until A_{600} reached 0.8. The broth was then added to 200 ml of the same medium, and the inoculated cells were grown at 37°C for 12 h. The cells were harvested by centrifugation for 10 min at 4°C and 8,400*g*, and then washed with 0.9% sodium chloride.

Site-Directed Mutagenesis of FHMPC Dehydrogenase—Genes encoding H137L and E149Q mutant FHMP dehydrogenases were prepared by essentially the same method as that described previously (*8*). The primers used were: for H137L, 5′-TGGGCATGCTCTGGTCGAA-3′ and 5′-TTCGACCAGAGCATGCCCA-3′, and for E149Q, 5′-CGATGATCCAGGTGATCG-3′ and 5′-CGATCACCTGGATCATCG-3′. The underlining indicates mismatched sites. The expressed mutant enzymes were purified by essentially the same method as that used for purification of the wild-type enzyme. Because the H137L enzyme showed no activity, SDS–PAGE (*23*) was performed to determine the fractions containing it for purification.

Preparation of mlr6793 Gene-Disrupted and Complemented Strains of M. loti—The chromosomal *mlr6793* gene was disrupted with a disruption plasmid by homologous recombination. The disruption plasmid was constructed as follows. A 1.2-kb fragment harbouring the entire coding sequence of the full-length *mlr6793* gene plus its 250 bp lower region was amplified by PCR with KOD-plus polymerase (Toyobo) and a pair of primers, 5′-TCTAGAATGATCCGAAATATCGCCATCATC-3′ (the underlining indicates a *Xba*I site) and 5′-AAGCTTAGAACCAGTTGCTTGGCCATGA-3′ (the underlining indicates a *Hind*III site), and then cloned into pTA2 to construct pTA2-6793. pTA2-6793 was digested with *Xba*I and *Hind*III, and then inserted into the *Xba*I/*Hind*III sites of pK18*mobsacB* to construct pK18-6793. The 1.8-kb tetracycline resistance gene (Tc^R) obtained from pKRP12 by digestion with *Sal*I was inserted into the SalI site of pK18-6793 to construct disruption plasmid pK18-6793::Tc^R. The disruption plasmid was transferred into *M. loti* by *E. coli* S17-1-mediated conjugation (*24*), and resulting disruptant mutants (*M. loti* Δ6793) were selected, as described previously (*3*). A complement plasmid, pBBad-6793, was constructed by introducing the *Xba*I/*Hin*dIII fragment derived from pET6793 into the *Xba*I/*Hin*dIII sites of pBBad22K. The *M. loti* Δ6793 cells were transformed with the complement plasmid by electroporation, as described previously (*3*). The *M. loti* wild-type, disruptant (Δ6793) and complement strains were subjected to a growth test on synthetic media containing pyridoxine, 4-pyridoxic acid, FHMPC and HMPDC as sole carbon and nitrogen sources, at 30°C for 2 weeks.

Purification of Recombinant FHMPC Dehydrogenase—Harvested cells (2.0 g obtained from 400 ml of the above-mentioned culture broth) were suspended in 20 ml of buffer A (20 mM sodium phosphate, pH 8.0, 10% glycerol and 0.01% 2-mercaptoethanol) supplemented with 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The cell suspension was sonicated on ice for 3 min with a Heatsystems Ultrasonicator W-220. A supernatant was obtained by centrifugation for 20 min at 4°C and 10,600*g*. The precipitate was re-suspended in 10 ml of the same buffer, and the suspension was sonicated and centrifuged again. The combined supernatant solution (27 ml) was used as the crude extract. The solution was applied to a QA52 column (2.8 × 10 cm; Whatman) equilibrated with buffer A. The column was washed with buffer A until A_{280} of the eluate become lower than 0.01. Then, the enzyme was eluted with a linear gradient with 0–0.3 M NaCl (250 ml each). The eluted enzyme solution (71 ml) was applied to a Blue A column (1.8 × 9.0 cm, Amicon) equilibrated with buffer A. The column was washed with buffer A and buffer A containing 1.5 M NaCl, as described above. Then, the enzyme was eluted with buffer A containing 5 mM NAD^+ and 1.5 M NaCl. The eluted solution (201 ml) containing the homogeneous enzyme protein was concentrated with a Vivaspin (Vivascience), and then dialysed against buffer A, and further applied to a PD-10 column (GE Healthcare) or a gel filtration column (COSMOSIL 5 Diol-300-II; Nacalai Tesque) equilibrated with buffer A to remove the NAD^+ used for the elution of the enzyme from the Blue A column.

Enzyme and Protein Assays—FHMPC dehydrogenase activity was usually determined by following the initial decrease in A_{380} (ε: 1000 M^{-1} cm^{-1}) due to FHMPC consumed at 30°C in 1 ml of a reaction mixture consisting of 0.1 M sodium phosphate, pH 8.0, 0.5 mM FHMPC, 0.5 mM cosubstrate (NAD^+ or NADH) and an appropriate amount of the enzyme. The absorbance at 380 nm was

measured because only FHMPC among substrates and products shows significant absorbance. One unit (U) was defined as the amount that catalysed the consumption of 1 μmol of FHMPC per min. The kinetic parameters for NAD^+ or NADH were determined in 0.1 M sodium phosphate buffer, pH 8.0, by varying the concentration of NAD^+ or NADH from 0 to 0.5 mM with a fixed FHMPC concentration (0.5 mM). The kinetic parameters for FHMPC were determined in the same buffer by varying the concentration of FHMPC from 0 to 0.5 mM with a fixed NAD^+ or NADH concentration (0.5 mM). Kinetic data were analysed with curve fitting software (Kaleida Graph) to fit the Michaelis–Menten equation with the Levenberg–Marquardt algorithm. The optimum pHs were determined in 100 mM buffers: sodium citrate (pH 4.5–5.5), MES (pH 5.5–7.0), sodium phosphate (pH 6.5–8.5) and TAPS (pH 8.0–9.0). The optimum pHs of wild-type and mutant enzymes were also determined in 100 mM GTA buffer (a universal buffer, pH 5.0–9.8), which consists of 33.3 mM (each) of 2,3-dimethylglutamate, Tris and 2-amino-2-methyl-1,3-propanediol. Protein was measured by the dye-binding method with bovine serum albumin (BSA) as the standard (*25*). The protein concentration of the purified enzyme was determined from the molecular absorption coefficient ($\varepsilon = 20{,}580\ M^{-1}\,cm^{-1}$ for the subunit) at 280 nm determined based on the amino acid composition (*26*).

Identification of Reaction Products—The reaction mixture (1 ml) comprising 50 mM sodium phosphate, pH 8.0, 1 mM FHMPC and 0.5 mM NAD^+ (or NADH) was preincubated for 10 min at 30°C. Then, the reaction was initiated by the addition of 0.2 U of FHMPC dehydrogenase. After 10, 40, 120 (or 180), 300 or 1,800 s, 100 μl of the reaction mixture was added to 10 μl of 60% $HClO_4$ to terminate the reaction. After centrifugation, the sample was filtrated through a filter membrane (DISMIC-13cp; Advantec) and diluted 50-fold. The samples and standards (4-pyridoxic acid, FHMPC and HMPDC) were quantitated by spectrophotometric analysis (*19*) and reversed-phase isocratic HPLC on a Cosmosil $5C_{18}$-MS-II column (4.6 × 250 mm; Nacalai Tesque) with UV detection (316 nm). The diluted sample (50 μl) was applied. The mobile phase buffer (pH 3.38 adjusted with $HClO_4$) consisted of 0.05% triethanolamine, 0.85% acetonitrile, 75 mM sodium dihydrogen phosphate and 75 mM sodium perchlorate, and a flow rate of 1 ml/min was used.

Determination of Dissociation Constants by Fluorescence Spectroscopy—To determine the dissociation constant of NADH, fluorescence titration was performed with a Hitachi Spectrofluorometer F-2000 at 25°C (*27*): 2.5 μl of NADH was added five times to 2.5 ml of 9.23–15.1 μM enzyme (subunit concentration) in 0.1 M sodium phosphate, pH 8.0. Controls were obtained by the same procedure in the same buffer without the enzyme. The excitation wavelength was set to 360 nm, and emission was measured between 400 and 600 nm. The maximal fluorescence enhancement of the binary Enzyme-NADH complex, ΔF_{max}, was determined with curve fitting software. The K_d value was obtained by linear regression analysis of $1/(1-\alpha)$ *versus* [NADH]/α, where α is equal to $\Delta F/\Delta F_{max}$ and the slope of the line is equal to $1/K_d$ (*28*). The dissociation constant of FHMPC was determined by the same method with a 0.6 μM enzyme (subunit concentration) solution. The excitation wavelength was set to 316 nm, and emission was measured between 300 and 600 nm. Each fluorescence measurement was performed in triplicate.

Other Analytical Methods—The molecular mass of the recombinant enzyme was determined by HPLC with size-exclusion chromatograph (COSMOSIL 5 Diol-300-II). A calibration curve was obtained based on the elution pattern of glutamate dehydrogenase (320 kDa, Oriental Yeast), catalase (240 kDa, Sigma), lactate dehydrogenase (140 kDa, Oriental Yeast), alanine aminotransferase (110 kDa, Sigma), malate dehydrogenase (72 kDa, Oriental Yeast), BSA (66 kDa, Sigma), carbonic anhydrase (29 kDa, Sigma) and cytochrome *c* (12.4 kDa, Sigma). The subunit molecular mass and purity of the enzyme were determined by SDS–PAGE (*23*) with Bio-Rad low-molecular-weight protein standards. The subunit molecular mass was also determined with an Applied Biosystems Voyager System 6366 MALDI-TOF MAS with α-cyano-4-hydroxy cinnamic acid as a matrix. Amino acid sequencing was performed with an Applied Biosystems 492 protein sequencer.

The acid dissociation constant (pK_a) was calculated from the optimum pH profile with a Hulinks enzyme kinetic module 1.3. DNA and protein databases in the DDBJ/EMBL/GenBank™ were searched for proteins homologous with FHMPC dehydrogenase by use of BLAST (*29*). Conserved domain of FHMPC dehydrogenase was searched for with PROSITE (http://us.expasy.org/prosite). Multiple sequence alignment was carried out using ClustalW (*30*). The tertiary structure of the enzyme was deduced with Geno3D (http://geno3d-pbil.ibcp.fr) with human 3-hydroxyacyl-CoA dehydrogenase as a model.

RESULTS

Identification, Cloning and Expression of the Gene Encoding FHMPC Dehydrogenase Involved in the Pyridoxine-Degradation Pathway—*Escherichia coli* BL21(DE3) cells transformed with pET6793 showed very high FHMPC dehydrogenase activity (49.8 U/mg of protein), while those transformed with the control plasmid (pET21a) showed no detectable activity. The crude extract of BL21(DE3)/pET6793 cells gave a dense protein band corresponding to the enzyme protein (Fig. 2I). The results showed that the *mlr6793* gene encoded FHMPC dehydrogenase.

To confirm that FHMPC dehydrogenase encoded by *mlr6793* gene is involved in the pyridoxine-degradation pathway, an *mlr6793*-disrupted strain of *M. loti* was prepared. The disruption of the *mlr6793* gene in the chromosome of *M. loti* Δ6793 cells was confirmed by PCR with the primers described above (Fig. 2II). Their growth on the synthetic medium containing pyridoxine, 4-pyridoxic acid, FHMPC or HMPDC as a sole carbon and nitrogen source was examined. *Mesorhizobium loti* wild-type cells could grow on the synthetic medium containing pyridoxine, 4-pyridoxic acid or FHMPC (Fig. 2III). In contrast, *mlr6793*-disruptant cells could

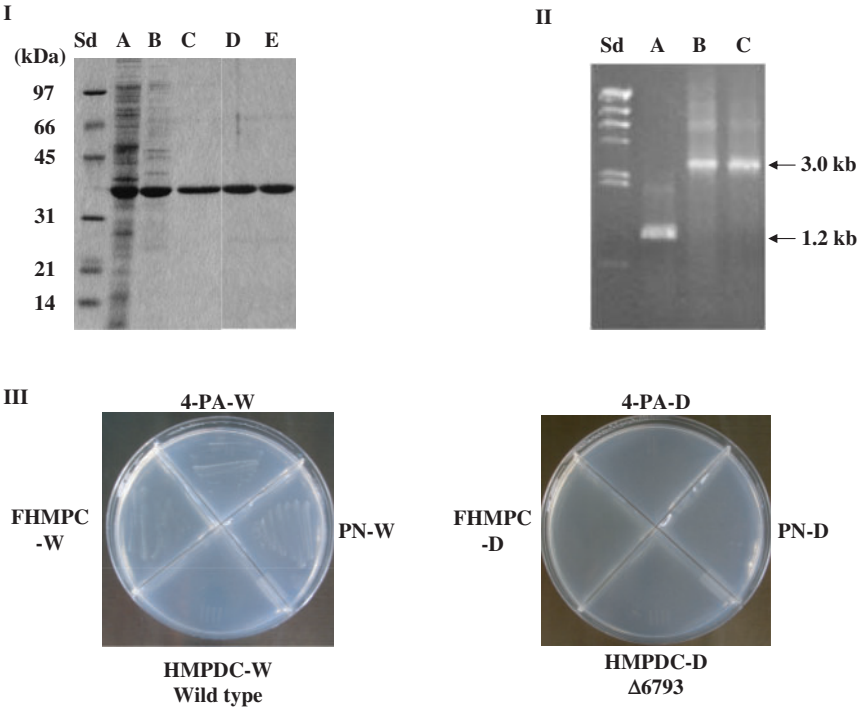


Fig. 2. **SDS–PAGE and agarose gel electrophoretic patterns, and growth of *M. loti* cells.** (I) SDS–PAGE patterns of preparations obtained at individual purification steps from BL21(DE3)/pET6793 cells and the purified mutant enzymes. Lane A, the crude extract (10 μg of protein); lane B, the purified fraction (5 μg) from the QA52 column; lane C, the purified fraction (2 μg) from the BlueA column; lane D, the purified H137L mutant enzyme (2 μg); lane E, the purified E149Q mutant enzyme (2 μg). The standard proteins (Bio-Rad SDS–PAGE Molecular Weight Standards, Low Range) were applied to lane Sd. (II) Agarose gel electrophoretic patterns. PCR amplification products obtained with the chromosomes of the wild-type (lane A) and disruptant (lane C) *M. loti* cells, and plasmid pK18-6793::Tc^R (lane B) as a template were applied to 1% agarose gel. As a molecular weight standard, lambda/*Hind*III (New England Biolabs) was used (lane Sd). (III) Growth of wild-type and *mlr6793*-disrupted *M. loti* cells on synthetic media. The wild-type and *mlr6793*-disrupted cells were cultivated for 2 weeks at 30°C on synthetic media containing 0.1 mM of pyridoxine (PN-W and PN-D), 4-pyridoxic acid (4-PA-W and 4-PA-D), FHMPC (FHMPC-W and FHMPC-D) or HMPDC (HMPDC-W and HMPDC-D), respectively.

not grow on any of them. The complement cells of the disruptant could grow in the same manner as the wild-type cells on the synthetic media (data not shown). The results showed that the enzyme encoded by *mlr6793* was essential for and involved in the degradation pathway. Interestingly, HMPDC was not assimilated by either the wild-type or mlr6793-disruptant cells, suggesting that it could not be transported across the bacterial cell membrane.

Purification and Properties of the Recombinant FHMPC Dehydrogenase—The recombinant enzyme was purified to homogeneity from BL21(DE3)/pET6793 cells through two column chromatography steps (Fig. 2I and Table 1). The purified enzyme gave a single band corresponding to a molecular mass of 38.9 ± 0.77 kDa on SDS–PAGE (Fig. 2I), which was about 6 kDa higher than that (33.06 kDa) of the subunit deduced from the nucleotide sequence of *mlr6793* for an unknown reason. Then, the molecular mass of the subunit was also determined by TOF-MS: the value was 33.121 kDa, coinciding with the deduced mass. The amino-terminal 15 amino acid sequence was MIRNIAIIGLGTMGP, which was identical with that deduced from the nucleotide sequence of *mlr6793*. The molecular mass of the native enzyme was determined to be 59.1 ± 2.02 kDa on the size-exclusion chromatography. The results showed that FHMPC dehydrogenase was a homodimeric protein. The enzyme had a tendency to polymerize into a dimer, tetramer or octamer in the absence of dithiothreitol or 2-mercaptoethanol. The polymers were enzymatically active like the native dimer. The purified enzyme showed a single absorption maximum at 279 nm when its absorption was monitored from 250 to 500 nm (data not shown), showing the absence of bound NAD^+ or NADH in the enzyme protein.

A plot of enzyme activity *versus* temperature gave a bell-shaped curve, and the optimum temperature was

Table 1. **Purification steps for recombinant FHMPC dehydrogenase.**

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Crude extract	177	8790	49.8	100
QA52	38.5	5140	133	58.5
BlueA	22.7	4530	199	51.5

The enzyme assay was performed in 1 ml of a reaction mixture consisting of 0.1 M sodium phosphate, pH 8.0, 0.5 mM FHMPC and 0.5 mM NAD^+ at 30°C. One unit (U) was defined as the amount that catalysed the consumption of 1 μmol of FHMPC per minute.

55°C in 100 mM sodium phosphate, pH 8.0; 29% and 36% of the maximal activity were observed at 30°C and 65°C, respectively. The activation energy calculated from an Arrhenius plot was 41.5 ± 1.4 kJ/mol.

The optimum pH of the enzyme reaction between FHMPC and NAD^+ or between FHMPC and NADH was determined in various kinds of buffer, it being pH 8.0 in 100 mM sodium phosphate buffer (data not shown). The optimum pH was also determined in the universal buffer to get a continuous curve, it being pH 8.5, as shown in Fig. 3A.

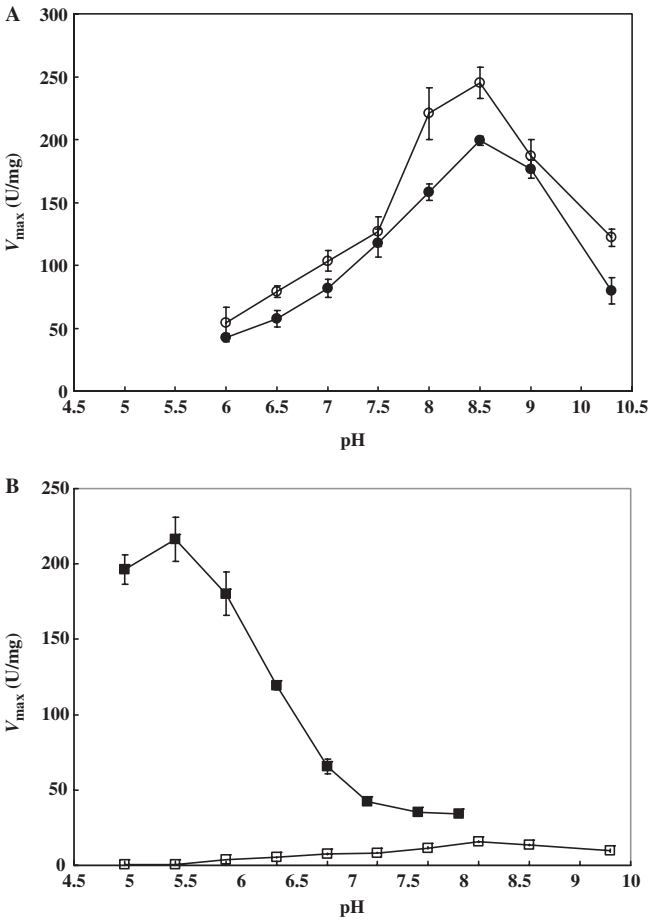


Fig. 3. **Effect of pH on the activity of the wild-type (A) or mutated (E149Q) (B) FHMPC dehydrogenase.** The V_{max} values were determined at 30°C in a reaction mixture consisting of FHMPC and NAD^+ (open symbols) or NADH (closed symbols) in 100 mM GTA buffer (pH 5.0–9.8).

The kinetic parameters determined in 100 mM sodium phosphate buffer, pH 8.0, with the combination of FHMPC and NAD^+ or that of FHMPC and NADH as substrates are shown in Table 2. The k_{cat} values of FHMPC, NAD^+ and NADH were almost the same with the two combinations. In contrast, the K_m values were not the same: those of NADH, FHMPC (in the combination with NADH), NAD^+ and FHMPC (in the combination with NAD^+) increased, in that order. Thus, the reaction efficiency (k_{cat}/K_m) for the substrates decreased in that order. The K_m value for FHMPC measured in the combination with NAD^+ was twice higher than that measured in the combination with NADH, suggesting that a conformation of the active site of the enzyme was affected by the binding of the cosubstrates.

The K_d values determined by the fluorescence titration method were 63.6 ± 9.1 μM for FHMPC, and 10.9 ± 0.6 for NADH. These values were comparable to their K_m values shown in Table 2.

The reaction products derived from FHMPC were determined at several reaction times. When the enzyme reaction was started with FHMPC and NAD^+ as substrates, almost equimolar amounts of HMPDC and 4-pyridoxic acid were produced (Table 2 and Fig. 4A): the ratio remained constant throughout the reaction (Fig. 4B). When the reaction was started with FHMPC and NADH instead of NAD^+, almost equimolar amounts of HMPDC and 4-pyridoxic acid were produced throughout the reaction like in the reaction with FHMPC and NAD^+ (data not shown). The ratio of HMPDC and 4-pyridoxic acid produced did not change when the enzyme reaction was performed in 100 mM sodium citrate buffer (pH 5.5), MES buffer (pH 6.0), 100 mM sodium phosphate buffer (7.0) or 100 mM TAPS buffer (pH 9.0). The results showed that FHMPC dehydrogenase catalysed the dismutation reaction.

FHMPC dehydrogenase did not reversibly reduce HMPDC (0.2 mM) to FHMPC with NADH (0.5 mM) even when an excess amount (0.12 U, 3.78 nM, about 100-fold higher than the amount used for the standard assay) of the enzyme was used. The enzyme (0.12 U) did not produce a measurable amount of FHMPC through a reversible oxidation of 4-pyridoxic acid with NAD^+ (0.5 mM), as shown previously (*13*), when the reaction was performed at 30°C for 1 h. The results showed that FHMPC dehydrogenase is practically a dismutase.

Amino Acid Sequence Comparison—The amino acid sequence of FHMPC dehydrogenase was compared with those of enzyme proteins whose substrate specificities have been determined. It showed low but significant

Table 2. **Kinetic parameters of the wild-type enzyme and E149Q mutant of FHMPC dehydrogenase determined in 100 mM sodium phosphate buffer, pH 8.0 or 100 mM sodium citrate buffer, pH 5.0.**

Enzyme	pH	Substrates	k_{cat} (s^{-1})	K_m (μM)		4-PA/HMPDC[a]
				FHMPC	NAD^+ or NADH	
Wild-type	8.0	FHMPC-NAD^+	204 ± 11.8	48.2 ± 9.9	34.3 ± 3.7	47/53
		FHMPC-NADH	217 ± 5.7	24.9 ± 3.3	12.4 ± 0.5	47/53
E149Q	8.0	FHMPC-NAD^+	25 ± 0.8	241.0 ± 27.0	5.8 ± 0.6	47/53
		FHMPC-NADH	78 ± 2.4	20.0 ± 0.0	26.0 ± 0.0	72/28
	5.0	FHMPC-NADH	300 ± 20.0	30.0 ± 4.2	33.0 ± 8.4	90/10[a]

[a]4-PA/HMPDC shows the relative amounts of 4-pyridoxic acid and HMPDC in the reaction mixture at final equilibrium.

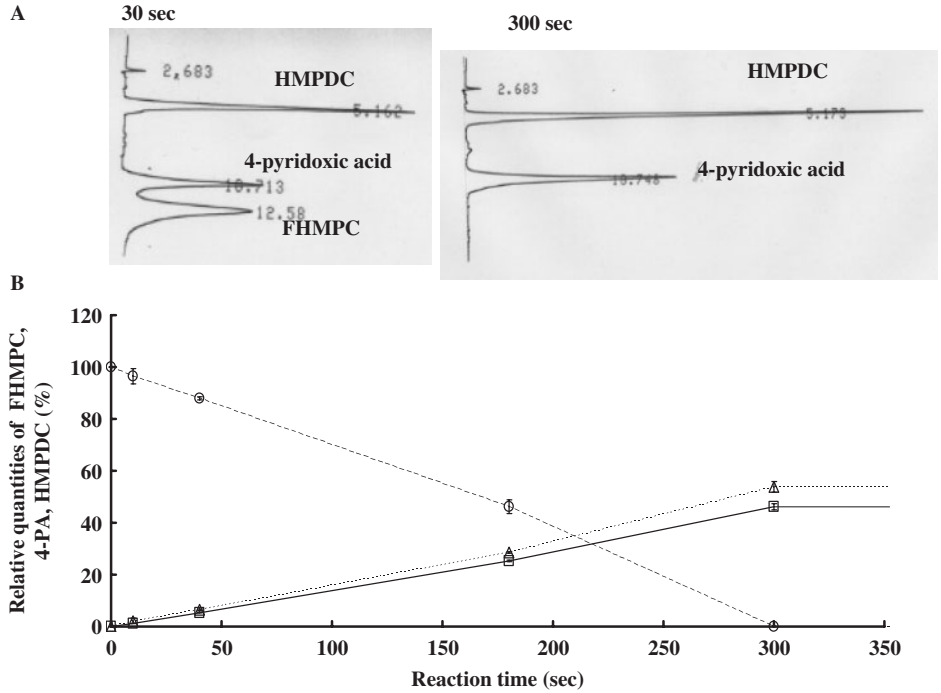


Fig. 4. **Reaction products arising during the FHMPC dehydrogenase reaction.** (A) HPLC analysis of the reaction products after 30- and 300- s-reaction. The reaction mixture (1 ml) comprising 100 mM sodium phosphate, pH 8.0, 1 mM FHMPC and 0.5 mM NAD^+ was pre-incubated for 10 min at 30°C. Then, the reaction was started by the addition of 0.2 U of FHMPC dehydrogenase. After 10, 40, 180 or 300 s, an aliquot (100 µl) of the reaction mixture was mixed with 10 µl of 60% $HClO_4$ to stop the reaction. After centrifugation, the sample was filtrated, diluted 50-fold and then subjected to HPLC. (B) Relative quantities (%) of FHMPC (circle, broken line), 4-pyridoxic acid (square, solid line) and HMPDC (triangle, dotted line) arising during the reaction.

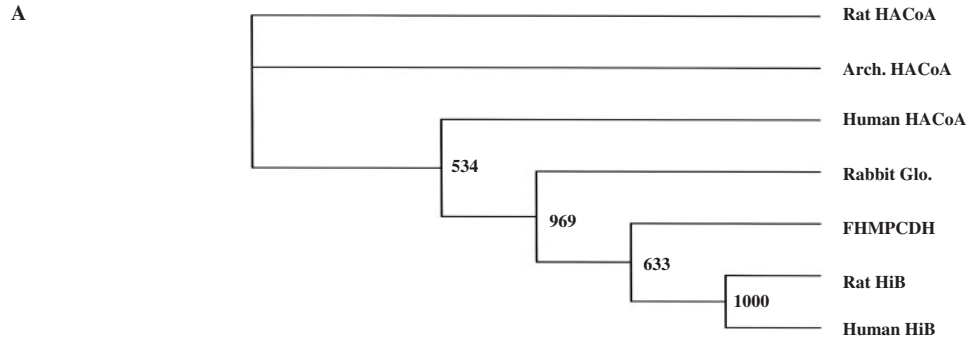
identity with those of rat and human 3-hydroxyisobutyrate dehydrogenases, rabbit L-gulonate 3-dehydrogenase and human, *Archaeoglobus* and rat L-3-hydroxyacyl-CoA dehydrogenases, as shown as a rectangular cladogram in Fig. 5A. In contrast, it showed no identity with those of general aldehyde dehydrogenases.

The amino acid sequences of enzyme and homologous enzymes were aligned (Fig. 5B). The enzyme showed two PROSITE motifs on its amino acid sequence: one at 7–20 (italic letters in Fig. 5B) of the 3-hydroxyisobutyrate dehydrogenase signature, and the other at 181-206 (small letters in Fig. 5B) of the 3-hydroxyacyl-CoA dehydrogenase signature. The predicted secondary structure of FHMPC dehydrogenase was more similar to that of human 3-hydroxyacyl-CoA dehydrogenase (code of Protein Data Bank, 1F12) than that of human 3-hydroxyisobutyrate dehydrogenase (2GF2). Furthermore, the histidine and glutamate residues (outlined letters in Fig. 5B) known to be involved directly in the catalysis of human 3-hydroxyacyl-CoA were conserved in FHMPC dehydrogenase, suggesting that these residues are involved in the catalytic reaction of FHMPC dehydrogenase.

Properties of Mutated FHMPC Dehydrogenase—The His137 and Glu149 residues were converted into Leu (H137L) and Gln (E149Q), respectively, in order to elucidate their contribution to the catalysis. The mutant enzymes were purified homogeneously as shown in Fig. 2I. The mutant enzymes were co-eluted with the wild-type enzyme on size-exclusion column chromatography, showing that they had the same molecular mass as that of the wild-type enzyme.

The H137L enzyme showed no measurable activity, showing that His137 is essential for the catalysis. The E149Q mutant enzyme showed an interesting property that provided a clue for predicting a reaction mechanism for FHMPC dehydrogenase, as described under 'Discussion' section; that is, it showed a different pH optimum depending on the co-substrate. When NAD^+ was used, the mutant enzyme showed very low activity with an optimum pH at 8.5 in the universal buffer (Fig. 3B). In contrast, the optimum pH was 5.5 when NADH was used in the same buffer (Fig. 3B). The E149Q mutant enzyme showed an optimum pH of 8.0 in 100 mM sodium phosphate buffer and one of 5.0 in 100 mM sodium citrate buffer when NAD^+ and NADH was used, respectively.

The kinetic parameters for the E149Q mutant enzyme are shown in Table 2. The mutant enzyme showed 12% of the k_{cat} values for FHMPC and NAD^+ compared to those (100%) of the wild-type one at pH 8.0. The mutation resulted in a 5-fold increase and a 6-fold decrease in the K_m values of FHMPC (in combination with NAD^+) and NAD^+ at pH 8.0, respectively. At pH 8.0, the mutant enzyme showed 37% of the k_{cat} values for FHMPC and NADH compared to those (100%) of the wild-type one. In contrast, at pH 5.0, the E149Q mutant enzyme showed 148% of the k_{cat} values for FHMPC and NADH



B

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Rat HiB          1 GHGAEAGRGAAWQPPWGSGGHASGLRYWSGRRRPVGSLAAVCSRSMASKTPVGFIGLGNMGNPMAKNLIKHGYPLILYDVFP--------DVCKEFKEAG-EQVASSPADVAEKADRIITMLPS 115
Human HiB        1 ------------------------------------------------MPVGFIGLGNMGNPMAKNLMKHGYPLIIYDVFP--------DACKEFQDAG-EQVVSSPADVAEKADRIITMLPT  66
FHMPCDH          1 ------------------------------------------MIRNIAIIGLGTMGPGMAARLARGGLQVVAYDVAP--------AAIERARSMLSVAETVLDALGIALPSAGVGTVRF  69
Human HACoA      1 ----------------------------SSSSTASASAKKIIVKHVTVIGGGLMGAGIAQVAAATGHTVVLVDQTEDILAKSKKGIEESLRKVAKKKFAENPKAGDECVEKTLSTIAT  90
Rat HACoA        1 ----------ASGQAKALQYAFFAEKSANKWSTPSGASWKTASAQPVSSVGVLGLGTMGRGIAISFARVGISVVAVESDP--------KQLDAAKKIITFTLEKEASRAHQNGQASAKPKLR 104
Arch.HACoA       1 ------------------------------XGSDKIHHHHHHHXKVFVIGAGLXGRGIAIAIASK-HEVVLQDVSE----------------------KALEAAREQIPEELLSKIE  63
Rabbit Glo       1 ------------------------------------MASPAAGDVLIVGSGLVGRSWAMLFASGGFRVKLYDIEP-----RQITGALENIRKEMKSLQQSGSLKGSLSAEEQLSLISS  77

Rat HiB        116 SMNSIEVYSGANGILKKVKK-GSLLIDSS-TIDPSVSKELAKEVEKMGAVFMDAPVSGGVGAARSGN-------LTFMVGGVENEFAAAQELLGCMGSNVLYCGAVGSGQSAKICNNMLLAISM 230
Human HiB       67 SINAIEAYSGANGILKKVKK-GSLLIDSS-TIDPAVSKELAKEVEKMGAVFMDAPVSGGVGAARSGN-------LTFMVGGVEDEFAAAQELLGCMGSNVVYCGAVGTGQAAKICNNMLLAISM 181
FHMPCDH         70 TDDIGDAVSGADLVIENVPENISIKADVYRTIDGLIGQDTIVASDTSGIPITKLQAHISYPERMVGMHWSNPPHIIPMIEVIAGEKTAPQTVATIR-DLIRSIGLLPVVVKKdvpgfvenrvly 192
Human HACoA     91 STDAASVVHSTDLVVEAIVENLKVKNELFKRLDKFAAEHTIFASNTSSLQITSIANATTRQDRFAGLHFFNPVPVMKLVEVIKTPMTSQKTFESLV-DFSKALGKHPVS-CKDTPGFIVNRLLV 212
Rat HACoA      105 FSSSTKELSTVDLVVEAVFEDMNLKKKVFAELSALCKPGAFLCTNTSALNVDDIASSTDRPQLVIGTHFFSPAHVMRLLEVIPSRYSSPTTIATVM-SLSKKIGKIGVV-VGNCYGFVGNRMLA 226
Arch.HACoA      64 FTTTLEKVKDCDIVXEAVFEDLNTKVEVLREVERLTNAP--LCSNTSVISVDDIAERLDSPSRFLGVHWXNPPHVXPLVEIVISRFTDSKTVAFVE----GFLRELGKEVVVCKGQSLVNRFNA 181
Rabbit Glo      78 CTNLAEAVEGVVHIQECVPENLDLKRKIFAQLDSIVDDRVVLSSSSSCLLPSKLFTGLAHVKQCIVAHPVNPPYYIPLVELVPHPETSPATVDRTH-ALMRKIGQSPVRVLKEIDGFVLNRLQY 200

Rat HiB        231 IGTAEAMNLGIRSGLDPKLLAKILNMSSGRCWSSDTYNPVPGVMDGVPSSNNYQGGFGTTLMAKDLG----------LAQDSATSTKTPILLGSVAHQIYRMMCSKGYSKKDFSSVFQYLREE 343
Human HiB      182 IGTAEAMNLGIRLGLDPKLLAKILNMSSGRCWSSDTYNPVPGVMDGVPSANNYQGGFGTTLMAKDLG----------LAQDSATSTKSPILLGSLAHQIYRMMCAKGYSKKDFSSVFQFLREE 294
FHMPCDH        193 allreavdlvergvIDPEDLDTCVSWGIGYKIAVIGPMALLDMAGLDIYKSVSSFLNADLSNRDDVA----------PMVLEKTSASKFGIKSGEGMFCYTPEQTKALQAERARKLVAVRRIL 305
Human HACoA    213 PYLMEAIRLYERGDASKEDIDTAMKLGAGYPMG----PFELLDYVGLDTTKFIVDGWHEMDAENPLHQPS----------PSLNKLVAENKFGKKTGEGFYKYKLE---------- 304
Rat HACoA      227 PYYNQGFFLLEEG-SKPEDVDGVL-EEFGFKMG----PFRVSDLAGLDVGWKIRKGQGLTGPSLPPGTPVRKRGNSRYSPLGMLCEAGRFGQKTGKGWYQYDKPLGRIHKPDDPWLSTFLSQYRE 345
Arch.HACoA     182 AVLSEASRXIEEG-VRAEDVDRVWKHHLGLLYTLFGPLGNLDYIGLDVAYYASLYLYKRFGDEKFKPP----------EWLQEKIKKGEVGVKAGKGIYEYGPKAYEERVERLKKLLRFLGLE- 293
Rabbit Glo     201 AIISEAWRLVEEGIVSPSDLDLVMSDGLGMRYAFIGPLETMHLNAEGMLSYSDRYSEGMKRVLKSFG----------SIPEFSGATVEKVNQAMCKKVPADPEHLAARREWRDECLKRLAKLK 313

Rat HiB        344 ETF---------- 346
Human HiB      295 ET---------- 296
FHMPCDH        306 EGRE-------- 309
Human HACoA      ---------- 
Rat HACoA      346 VHHIEQRTISKEEILERCLYSLINEAFRILEEGMAARPEHIDVIYLHGYGWPRHKGGPMFYAASVGLPTVLEKLQKYYRQNPDIPQLEPSDYLRRLVAQGSPPLKEWQSLAGPHGSKL 463
Arch.HACoA       ---------- 
Rabbit Glo     314 RQMQPQ-------- 319
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Fig. 5. **Rectangular cladogram and multiple amino-acid sequence alignment of FHMPC dehydrogenase and homologous enzymes.** (A) Rectangular cladogram drawn with Clustal W. The sequence of FHMPC dehydrogenase (FHMPCDH) was compared with those of 3-hydroxyacyl-CoA dehydrogenase from rat (Rat HACoA, 1ZCJ), *Archaeoglobus fulgidus* (Arch. HACoA, 1ZEJ) and man (Human HACoA, 1F12), L-gulonate 3-dehydrogenase from rabbit (Rabbit Glo, 2DPO), and 3-hydroxyisobutyrate dehydrogenase from rat (Rat HiB) and man (Human HiB, 2GF2). (B) Amino acid sequence alignment. Histidine and glutamate residues involved directly in the catalysis are shown by outlined letters. The PROSITE motifs of the 3-hydroxyisobutyrate dehydrogenase and 3-hydroxyacyl-CoA dehydrogenase signatures are shown by italic and small letters, respectively. Secondary structures (α-helix, dotted underline, and β-sheet, underlined) are shown. Those of FHMPC dehydrogenase are the deduced ones.

compared to those (100%) of the wild-type enzyme at pH 8.0, showing that the mutation increased the reactivity of FHMPC dehydrogenase at the acidic pH when NADH was used as the co-substrate. The pK_a value of the residue involved in the catalysis was identified to be 6.7 from the pH-reaction profile of the E149Q mutant enzyme, suggesting that a His residue with a pK_a of 6.7 is involved in the catalysis in a protonated form. Since His137 is likely located in the active site as described later (Fig. 6A), His137 is presumably assigned to the catalytic residue. In the wild-type enzyme, a residue with a calculated pK_a of 6.7 is also involved in the catalysis in a deprotonated form (Fig. 3A), which may be assigned to His137. Thus, the protonation state of His137 is significantly affected by the presence of Glu149. The E149Q mutant enzyme showed no

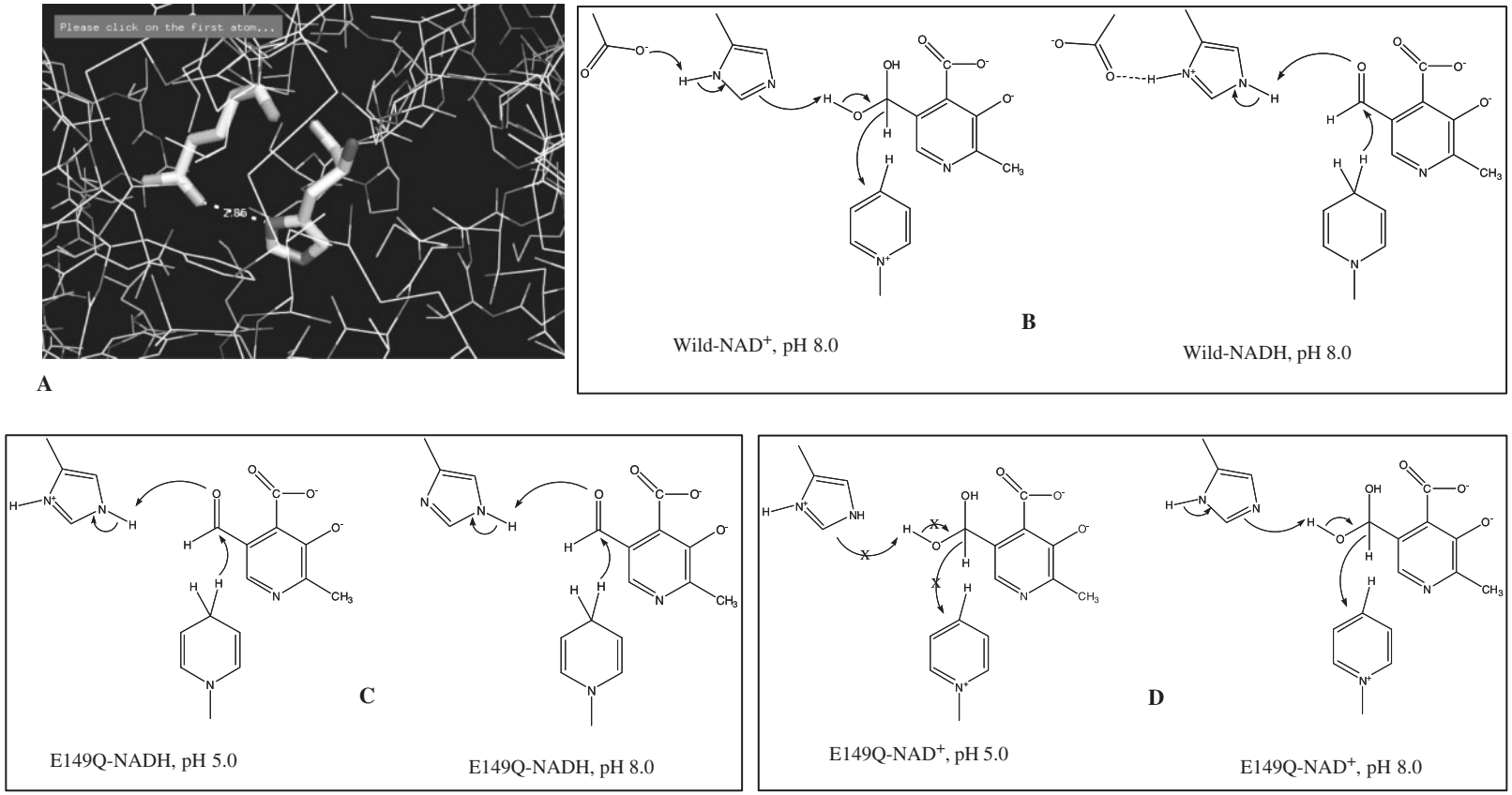

Fig. 6. **Deduced reaction mechanism for FHMPC dehydrogenase catalysis.** (A) Deduced distance between His137 and Glu149. (B) Reaction mechanism for the wild-type enzyme at pH 8.0. Glu149 assists the reaction with NAD^+ and NADH. (C) Reaction mechanism for the E149Q enzyme with NADH at pH 5.0 and 8.0. (D) Reaction mechanism for the E149Q enzyme with NAD^+ at pH 5.0 and 8.0.

measurable activity at pH 5.0 when NAD^+ instead of NADH was used as the cosubstrate.

The reaction products of the E149Q mutant enzyme were determined by isocratic-HPLC. The final amounts of the reaction products derived through the reaction between FHMPC and NAD^+ at pH 5.5–8.0 were 53% of HMPDC and 47% of 4-pyridoxic acid. On the contrary, those derived through the reaction between FHMPC and NADH at pH 8.0 were 28% of HMPDC and 72% of 4-pyridoxic acid. The proportion of 4-pyridoxic acid was increased at acidic pH (90% at pH 5.0).

The E149Q mutant enzyme was stable like the wild-type one: it showed no loss of activity after incubation at 37°C for 5 h in 100 mM sodium phosphate buffer, pH 8.0, in contrast to the corresponding human 3-hydroxyacyl-CoA dehydrogenase mutant (*18*).

DISCUSSION

The results showed that the *mlr6793* gene encodes FHMPC dehydrogenase (dismutase), which is involved in the degradation pathway for pyridoxine. The gene has been reported to encode 4-pyridoxic acid dehydrogenase, which catalyses the step before the reaction of FHMPC dehydrogenase (*12*). In this study, an extremely large amount (5 μM) of the recombinant enzyme protein was used for determination of the 4-pyridoxic acid oxidation activity. Such a large amount of recombinant FHMPC dehydrogenase could catalyse the oxidation of 4-pyridoxic acid to FHMPC, although the turnover number ($0.01\ s^{-1}$) was about 10,000-fold lower than that for the reduction of FHMPC to 4-pyridoxic acid or the oxidation of FHMPC to HMPDC. Thus, the results are consistent with the judgement that FHMPC dehydrogenase is practically a dismutase. In *M. loti* cells, there is a genuine 4-pyridoxic acid dehydrogenase, *i.e*. a membrane-bound enzyme involved in the degradation pathway (*5*).

The primary and deduced secondary structures of FHMPC dehydrogenase showed the highest homology to those of human 3-hydroxyacyl-CoA dehydrogenase (Fig. 5). His158 and Glu170 of human 3-hydroxyacyl-CoA dehydrogenase (*18*) located in its active site were conserved in FHMPC dehydrogenase: the corresponding residues were His137 and Glu149, respectively. His137 was essential for the catalysis, as described previously. In contrast, Glu149 played an assisting role in the catalysis. The residues in the predicted tertiary structure with human 3-hydroxyacyl-CoA dehydrogenase as a model are close (the distance between $N\delta^1$ of the imidazole ring and Oε of the γ-carboxyl group is 2.86 Å) and can form a catalytic diad (Fig. 6A). The E149Q mutant enzyme was stable in contrast to the E170Q mutant of human 3-hydroxyacy-CoA dehydrogenase, in which Glu170 forming a diad with His158 is reported to be required for proper orientation of His158 and structural integrity of the enzyme (*18*, *31*, *32*). Thus, Glu149 in FHMPC dehydrogenase appears to contribute to the enzyme catalysis in a different manner from that in the case of Glu170 in human 3-hydroxyacyl-CoA dehydrogenase, although the two glutamate residues can form the catalytic diad at the active sites of the enzymes.

Glu149 in FHMPC dehydrogenase seems to be involved in the catalysis. Thus, the catalysis by the wild-type FHMPC dehydrogenase at the optimum pH of 8.0 and in 100 mM sodium phosphate buffer can be depicted as shown in Fig. 6B, based on the general reaction scheme of NAD^+-dependent dehydrogenases (*33*). His137 serves as a general acid/base, with the catalysis being facilitated by the presence of Glu149. NAD^+ would oxidize a hemiacetal form of FHMPC to HMPDC. On the other hand, NADH would reduce an aldehyde form of FHMPC to 4-pyridoxic acid. The presence of Glu149 is necessary to drive the reaction at pH 8.0. Thus, the role of Glu149 was clearly demonstrated with the E149Q mutant enzyme. This mutant enzyme, which has the His137 catalytic residue with pK_a 6.7, can catalyse reduction of the aldehyde form of FHMPC in sodium citrate buffer, pH 5.0, with a higher k_{cat} value than that of the wild-type enzyme because the imidazole ring of His137 should be almost fully (about 98%) protonated at pH 5.0 and able to attract electrons, as shown in Fig. 6C. On the contrary, the mutant enzyme cannot oxidize FHMPC with NAD^+ at pH 5.0 because the protonated imidazole is not able to donate electrons to drive the oxidation reaction, as shown in Fig. 6D, without donation of electrons from Glu149. At pH 8.0, about 95% of the imidazole ring of His137 is in the deprotonated form, and it can catalyse the oxidation and reduction of FHMPC, as shown in Fig. 6C and D, although the rates are low because of no assistance by Glu149. Thus, the presence of Glu149 increased the k_{cat} values of the oxidation and reduction reactions of FHMPC about 10-fold and 3-fold, respectively, at pH 8.0 (Table 2).

FHMPC dehydrogenase is a NAD^+-dependent dismutase. What is a possible physiological significance of the enzyme reaction for *M. loti* cells? One possibility has been suggested by Snell *et al*. (*13*). Since 4-pyridoxic acid is the substrate for 4-pyridoxic acid dehydrogenase that is a membrane-bound enzyme, reconverts 4-pyridoxic acid to FHMPC, and can use CoQ as an electron acceptor for donating electrons to an electron transport chain to produce ATP (*14*), action of dismutase could serve to simplify the pathway for PN degradation in the cells by reducing the number of inducible dehydrogenases that require separate coupling to oxygen to permit oxidative energy to be conserved. Other possibilities such as saving loss of NAD^+ and NADH may be the case. Further study, including kinetics, is required to elucidate a mechanism and physiological role of the dismutation reaction catalysed by FHMPC dehydrogenase.

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CONFLICT OF INTEREST

None declared.

REFERENCES

1. 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 B6. *FEMS Microbiol. Lett.* **234**, 225–230
2. 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
3. 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
4. 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
5. Ge, F., Yokochi, N., Yoshikane, Y., Ohnishi, K., and Yagi, T. (2008) Gene identification and characterization of the pyridoxine degradative enzyme 4-pyridoxic acid dehydrogenase from the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti* MAFF303099. *J. Biochem.* **143**, 603–609
6. 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-zdioxygenase-coding gene from the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *J. Biosci. Bioeng.* **102**, 504–510
7. Yuan, B, Yokochi, N., Yoshikane, Y., Ohnishi, K., Ge, F., and Yagi, T. (2008) Gene identification and characterization of the pyridoxine degradative enzyme alpha-(N-acetylaminomethylene)succinic acid amidohydrolase from *Mesorhizobium loti* MAFF303099. *J. Nutr. Sci. Vitaminol.* **54**, 185–190
8. 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
9. 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
10. 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
11. Yagi, T., Yokochi, N., Yoshikane, Y., Yuan, B., Funami, J., Tadokoro, M., and Ge, F. (2005) Genes encoding enzymes involved in degradation for vitamin B6. Abstract of IUBMB Symposium, **L11R**
12. 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
13. Lee, Y.C., Nelson, M.J., and Snell, E.E. (1986) Enzymes of vitamin B_6 degradation. Purification and properties of isopyridoxal dehydrogenase and 5-formyl-3-hydroxy-2-methylpyridine-4-carboxylic-acid dehydrogenase. *J. Biol. Chem.* **261**, 15106–15111
14. 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
15. Henehan, G.T. and Oppenheimer, N.J. (1993) Horse liver alcohol dehydrogenase-catalyzed oxidation of aldehydes: dismutation precedes net production of reduced nicotinamide adenine dinucleotide. *Biochemistry* **32**, 735–738
16. Svensson, S., Lundsjö, A., Cronholm, T., and Höög, J-O. (1996) Aldehyde dismutase activity of human liver alcohol dehydrogenase. *FEBS Lett.* **394**, 217–220
17. LeBrun, L.A., Park, D-H., Ramaswamy, S., and Plapp, B.V. (2004) Participation of histidine-51 in catalysis by horse liver alcohol dehydrogenase. *Biochemistry* **43**, 3014–3026
18. Barycki, J.J., O'Brien, L.K., Strauss, A.W., and Banaszak, L.J. (2000) Sequestration of the active site by interdomain shifting. Crystallographic and spectroscopic evidence for distinct conformations of L-3-hydroxyacyl-CoA dehydrogenase. *J. Biol. Chem.* **275**, 27186–27196
19. Burg, R.W., Rodwell, V.W., and Snell, E.E. (1960) Bacterial oxidation of vitamin B6. II. Metabolites of pyridoxamine. *J. Biol. Chem.* **235**, 1164–1169
20. Schäfer, A., Tauch, A., Jäger, W., Kalinowski, K., Thierbach, G., and Pühler, A. (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**, 69–73
21. Reece, K.S. and Phillips, G.J. (1995) New plasmids carrying antibiotic-resistance cassettes. *Gene* **165**, 141–142
22. Sukchawalit, R., Vattanaviboon, P., Sallabhan, R., and Mongkolsuk, S. (1999) Construction and characterization of regulated L-arabinose-inducible broad host range expression vectors in *Xanthomonas*. *FEMS Microbiol. Lett.* **181**, 217–223
23. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
24. Simon, R., Priefer, U., and Puhler, A. (1983) A broad host range mobilization systems for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technol.* **1**, 784–791
25. 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. Biochem.* **72**, 248–254
26. Gill, S.C. and Hippel, P.H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319–326
27. Hurley, T.D., Edenberg, H.J., and Bosron, W.F. (1990) Expression and kinetic characterization of variants of human beta 1 beta 1 alcohol dehydrogenase containing substitutions at amino acid 47. *J. Biol. Chem.* **265**, 16366–16372
28. Holbrook, J.J. and Stinson, R.A. (1973) The use of ternary complexes to study ionizations and isomerizations during catalysis by lactate dehydrogenase. *Biochem. J.* **131**, 739–748
29. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402
30. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **22**, 4673–4680
31. Barycki, J.J., O'Brien, L.K., Bratt, J.M., Zhang, R., Sanishvili, R., Strauss, A. W., and Banaszak, L.J. (1999) Biochemical characterization and crystal structure determination of human heart short chain L-3-hydroxyacyl-CoA dehydrogenase provide insight into catalytic mechanism. *Biochemistry* **38**, 5786–5798
32. He, X-Y. and Yang, S-Y. (1996) Histidine-450 is the catalytic residue of L-3-hydroxyacyl coenzyme A dehydrogenase associated with the large alpha-subunit of the multienzyme complex of fatty acid oxidation from *Escherichia coli*. *Biochemistry* **35**, 9625–9630
33. Bugg, T.D.H. (2004) *Introduction to Enzyme and Coenzyme Chemistry*, pp. 132–133, Blackwell Publishing Ltd., Oxford