Identification and Characterization of D-Hydroxyproline Dehydrogenase and Δ^1-Pyrroline-4-hydroxy-2-carboxylate Deaminase Involved in Novel L-Hydroxyproline Metabolism of Bacteria

METABOLIC CONVERGENT EVOLUTION

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Background: The bacterial pathway of L-hydroxyproline metabolism has not been identified.

Results: Different types of D-hydroxyproline dehydrogenases and unique Δ^1-pyrroline-4-hydroxy-2-carboxylate deaminase involved in the bacterial L-hydroxyproline pathway were identified and characterized for the first time.

Conclusion: L-Hydroxyproline degradation by bacteria was elucidated at the molecular level.

Significance: Our results suggest that D-hydroxyproline dehydrogenases evolved convergently, and we discovered a unique deaminase enzyme likely within the aldolase protein family.

L-Hydroxyproline (4-hydroxyproline) mainly exists in collagen, and most bacteria cannot metabolize this hydroxyamino acid. Pseudomonas putida and Pseudomonas aeruginosa convert L-hydroxyproline to α-ketoglutarate via four hypothetical enzymatic steps different from known mammalian pathways, but the molecular background is rather unclear. Here, we identified and characterized for the first time two novel enzymes, D-hydroxyproline dehydrogenase and Δ^1-pyrroline-4-hydroxy-2-carboxylate (Pyr4H2C) deaminase, involved in this hypothetical pathway. These genes were clustered together with genes encoding other catalytic enzymes on the bacterial genomes. D-Hydroxyproline dehydrogenases from P. putida and P. aeruginosa were completely different from known bacterial proline dehydrogenases and showed similar high specificity for substrate (D-hydroxyproline) and some artificial electron acceptor(s). On the other hand, the former is a homomeric enzyme containing only FAD as a prosthetic group, whereas the latter is a novel heterododecameric structure consisting of three different subunits (α,β,γ), and two FADs, FMN, and [2Fe–2S] iron-sulfur cluster were contained in αβγ of the heterotrimeric unit. These results suggested that the L-hydroxyproline pathway clearly evolved convergently in P. putida and P. aeruginosa. Pyr4H2C deaminase is a unique member of the dihydrodipicolinate synthase/N-acetylneuraminic lyase protein family, and its activity was competitively inhibited by pyruvate, a common substrate for other dihydrodipicolinate synthase/N-acetylneuraminic lyase proteins. Furthermore, disruption of Pyr4H2C deaminase genes led to loss of growth on D-hydroxyproline (as well as D-hydroxyproline) but not L- and D-proline, indicating that this pathway is related only to L-hydroxyproline degradation, which is not linked to proline metabolism.

D-Hydroxyproline (4-hydroxyproline) exists in collagen and the cell wall of plants and algae. Although direct biosynthesis from L-proline has been known in a few bacteria (1), the free compound in nature is mainly produced from collagen by amino peptidase(s). Metabolism of L-hydroxyproline in mammals has been well studied and involves the stepwise action of four mitochondrial enzymes (see Fig. 1A) (2). L-Hydroxyproline oxidase (so-called PRODH2) first produces Δ^1-pyrroline-3-carboxylate (Pyr3H5C). Pyr3H5C is a spontaneously opened ring and is converted to L-4-hydroxy-5-carboxylate (Pyr5H5C).

The abbreviations used are: Pyr3H5C, Δ^1-pyrroline-3-carboxylate; Pyr5H5C, Δ^1-pyrroline-5-carboxylate; T. profundus, Thermus profundus; Pf/L-PDH1, L-PDH isozyme 1 of P. horikoshii; Pf/L-PDH2, L-PDH isozyme 2 of P. horikoshii; Pyr4H2C, Δ^1-pyrroline-4-hydroxy-2-carboxylate; Δ-HypDH, D-hydroxyproline dehydrogenase; αKGS, α-ketoglutaric semialdehyde; KGSADH, αKGS dehydrogenase; Pf/L-PDH from P. putida; Pa/Δ-HypDH, Δ-HypDH from P. aeruginosa; Hcn, hydrogen cyanide synthase; Nox, n-octopine oxidase; Oxx, α-octopine oxidase; Pa, P. putida; Pa, P. aeruginosa; Ab, A. brasilense; Km, kanamycin resistance; INT, p-iodonitrotetrazolium violet; PMS, phenazine methosulfate; NBT, nitroblue tetrazolium; l-KDA, l-2-keto-3-deoxyarabinonate.

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oxoglutarate by the sequential action of aspartate aminotransferase (EC 2.6.1.1) and 4-hydroxy-2-oxoglutarate aldolase (EC 4.1.3.16). Recently, PRODH2 and 4-hydroxy-2-oxoglutarate aldolase were characterized genetically and enzymatically (3, 4).

The mammalian L-hydroxyproline pathway is partially linked to the general L-proline pathway in which L-proline is metabolized to L-glutamate by two enzymes (see Fig. 1B). The first enzyme, L-proline dehydrogenase (L-PDH; EC 1.5.99.8), catalyzes the flavin adenine dinucleotide (FAD)-dependent oxidation of L-proline to Pyr5C. It is also known that Pyr5C reductase (EC 1.5.1.12) can reduce either Pyr5C or Pyr3H5C to L-proline or L-hydroxyproline, respectively (5). The Pyr5C intermediate is spontaneously hydrolyzed to glutamate/γ-semialdehyde, which is subsequently oxidized to L-glutamate by the second enzyme, Pyr5C dehydrogenase. Intriguingly, whereas L-PDH and Pyr5C dehydrogenase are separate enzymes in eukaryotes and some bacteria (6), they are fused into a single polypeptide chain in other bacteria that is known as PutA (proline utilization A) (7). On the other hand, three L-PDHs from hyperthermophilic archaea differing from the known L-PDHs have been recently reported, although the reaction product is the same, Pyr5C (summarized in supplemental Table S6). First, L-PDH of Thermococcus profundus (Tp/L-PDH) forms a complex consisting of four different subunits with molecular masses of 54 (α), 42 (β), 19 (γ), and 11 kDa (δ), respectively, with a heterotetrameric structure of αβγδ containing two FADs (in α- and β-subunits) (8). Second, L-PDH isozyme 1 of Pyrococcus horikoshii (Ph/L-PDH1) consists of two different subunits with molecular masses of 56 (α) and 43 kDa (β) with a heterooctameric structure of α2β2 containing one FAD (β), one FMN (between α- and β-subunits), and one ATP (α) (9, 10). P. horikoshii also possesses L-PDH isozyme 2 (Ph/L-PDH2), which is similar to Tp/L-PDH. Third, L-PDH of Pyrobaculum calidifontis is a homodimeric enzyme containing one FAD per subunit (11). These enzymes can utilize artificial electron acceptors such as 2,6-dichloroindophenol and ferricyanide instead of the natural acceptors.

Although L-hydroxyproline is a non-utilizable amino acid for most microorganisms, a few bacteria, including Pseudomonas putida (12), Pseudomonas aeruginosa (13, 14), and Pseudomonas striata (15, 16), can rapidly grow on L-hydroxyproline as a sole carbon source, and it is believed that the hypothetical pathway, different from that in mammals, is operational (Fig. 1C). The first two steps in this pathway involve epimerization to D-hydroxyproline catalyzing L-hydroxyproline 2-epimerase (EC 5.1.1.8) and oxidation to \( \Delta^1 \)-pyrroline-4-carboxylate (Pyr4H2C) catalyzing D-hydroxyproline dehydrogenase (oxidase) (d-HypDH; EC 1.4.3.-). The cyclic imine Pyr4H2C is spontaneously hydrolyzed to 4-hydroxy-2-oxo-5-aminovalerate, which is subsequently deaminated to α-ketoglutaric acid (HOG aldolase; EC 4.1.3.16) with the production of glyoxylic acid and glutamate (6).
semialdehyde (αKGSA) by Pyr4H2C deaminase (EC 3.5.4.22). The last step is the NAD(P)⁺-dependent dehydrogenation of the semialdehyde to α-ketoglutarate by αKGSA dehydrogenase (KGSADH) (2,5-dioxovalerate dehydrogenase; EC 1.2.1.26). The αKGSA intermediate is also produced from alternative pentose and/or acid-sugar pathways of bacteria and/or archaea (17–19), and the metabolic fate is the same as that of the bacterial L-hydroxyproline pathway. Previously, we first identified three KGSADH isozymes by L-arabinose (KGSADH-I), D-glucarate/D-galactarate (KGSADH-II), and L-hydroxyproline (KGSADH-III; LhpG in this study) in Azospirillum brasilense, a nitrogen-fixing bacteria (20, 21).

Although genetic and enzymatic characterizations of L-hydroxyproline 2-epimerases from several bacteria, including P. aeruginosa, were recently reported (4), only the genes encoding D-HypDH and Pyr4H2C deaminase have been identified so far. In particular, FAD-dependent amino-acid dehydrogenases, including D-HypDHs from P. aeruginosa (13) and P. striata (15), generally associate with cell and/or organelle membranes and are unstable in solution, which has made purification, preservation, and characterization very difficult. In this study, we identified different types of putative gene clusters related to L-hydroxyproline metabolism of P. putida and P. aeruginosa by bioinformatics analysis. Furthermore, characterization of the recombinant proteins expressed in P. putida or Escherichia coli cells revealed that D-HypDHs from P. putida and P. aeruginosa (referred to as Pp/D-HypDH and Pa/D-HypDH, respectively) had clearly evolved convergently and that Pyr4H2C deaminase belongs to a unique member of the protein family consisting of aldolase proteins.

MATERIALS AND METHODS

General Procedures

Basic recombinant DNA techniques were performed as described by Sambrook et al. (23). Bacterial genomic DNA was prepared using a DNeasy Tissue kit (Qiagen). PCR was carried out using GeneAmp PCR System 2700 (Applied Biosystems) for 30 cycles in a 50-μl reaction mixture containing 1 unit of KOD FX DNA polymerase (Toyobo), 15 pmol of the appropriate primers, and template DNA under the following conditions: denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and extension at 68 °C for time periods calculated as an extension rate of 1 kbp/min. Protein concentrations were determined by the method of Lowry et al. (24) with bovine serum albumin as the standard. SDS-PAGE was performed as described by Laemmli (25). Non-denaturing PAGE was performed by omitting SDS and 2-mercaptoethanol from the solution used in SDS-PAGE. Proteins on the gel were stained with Coomassie Brilliant Blue R-250 and destained with 7.5% (v/v) acetic acid in 25% methanol. High pressure liquid chromatography (HPLC) was performed using an Agilent 1120 Compact LC system (Tosoh), NMR spectra were recorded on a Jeol JNM-EC400 NMR spectrometer (Jeol Ltd., Tokyo, Japan) operating at 400 MHz. 2,2-Dimethyl-2-silapentane-5-sulfonate was used as an internal standard.

Bacterial Strain, Culture Conditions, and Preparation of Cell-free Extracts

Pseudomonas strains were cultured aerobically with vigorous shaking at 30 °C in LB medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl/liter) or minimal medium (pH 6.8) containing 0.8 g of KH₂PO₄, K H₂PO₄, 2 g of (NH₄)₂SO₄, 0.002 g of FeSO₄, 0.080 g of MgSO₄·7H₂O, and 2 g of carbon source/ liter. The grown cells were harvested by centrifugation at 30,000 × g for 20 min, suspended in 50 mM Tris-HCl (pH 8.0) containing 2 mM MgCl₂, and disrupted by sonication for 20 min at appropriate intervals on ice using an Ultra Sonic Disruptor Model UR-200P (Tomy Seiko Co., Ltd, Tokyo, Japan) and then centrifuged at 108,000 × g for 20 min at 4 °C to obtain cell-free extracts. If necessary, 1% (v/v) Tween 20 was added to the buffer for solubilization of d-HypDH.

Plasmid Construction for Expression of Recombinant Proteins

Primer sequences used in this study are shown in supplemental Table S1. In this report, the prefixes P. putida (Pp), P. aeruginosa (Pa), and A. brasilense (Ab) have been added to gene symbols or protein designations when required for clarity. PpLhpB (PP_1255) PaLhpB-LhpF-LhpE (PA1266 and PA1267), PaLhpC (PP_1257), and PpLhpG (PP_1256) (GenBank™ accession numbers are given in parentheses) genes were amplified by PCR using primers containing appropriate restriction enzyme sites at the 5′- and 3′-ends and genomic DNA of P. putida KT2442 or P. aeruginosa PA01 as a template. In the case of the PaLhpB-FE gene, SacI and HindIII sites were attached to the 5′-end of the PaLhpB gene and the 3′-end of the PaLhpE gene, respectively. Each amplified DNA fragment was introduced into BamHI-HindIII sites of the plasmid pUCP26Km to yield pUCP26KmAhpCp. alkyl hydroperoxide reductase was amplified and introduced into SalI-EcoRI sites in pUCP26KmAhpCp to yield plasmid pUCP/PpLhpB and pUCP/PaLhpBFE, respectively, and genomic DNA of P. putida KT2442 or P. aeruginosa PA01 as a template. In the case of the PaLhpB-FE gene, SacI and HindIII sites were attached to the 5′-end of the PaLhpB gene and the 3′-end of the PaLhpE gene, respectively. Each amplified DNA fragment was introduced into BamHI-HindIII sites of the plasmid pUCP26Km to yield pUCP26KmAhpCp. The promoter and partial open reading frame region (~240 bp) of the ahpC gene from P. putida that encodes a small subunit of alkyl hydroperoxide reductase was amplified and introduced into the Sall site in pUCP26Km to yield plasmid pUCP26KmAhpCp. Each DNA fragment of His₆-PpLhpB-terminator and His₆-PaLhpBFE-terminator was amplified by PCR using pQE/PpLhpB and pQE/PaLhpBFE as a template, respectively, and introduced into Sall-EcoRI sites in pUCP26Km to obtain pUCP/PpLhpB and pUCP/PaLhpBFE, respectively (see Fig. 2F).

Expression and Purification of His₆-tagged Recombinant Proteins

PpLhpC and PpLhpG genes were expressed in E. coli cells, whereas PpLhpB and PaLhpBFE genes were expressed in P. putida cells. E. coli DH5α harboring pQE/PpLhpC or pQE/PpLhpG was grown at 37 °C to a turbidity of 0.6 at 600 nm in Super broth medium (pH 7.0; 12 g of tryptone, 24 g of yeast extract, 5 ml of glycerol, 3.81 g of KH₂PO₄, and 12.5 g of NaCl).
K$_2$HPO$_4$/liter) containing 50 mg/liter ampicillin. After the addition of 1 mM isopropyl β-D-thiogalactopyranoside, the culture was further grown for 6 h to induce the expression of His$_6$-tagged protein. On the other hand, _P. putida_ KT2442-oxyR1 (28) harboring pUCP/PpLhpB or pUCP/PaLhpBFE obtained using the the heat-shock transformation method was grown at 30 °C overnight in LB medium containing 50 mg/liter rifampicin and 50 mg/liter kanamycin. Cells were harvested and resuspended in Buffer A (50 mM sodium phosphate buffer (pH 8.0) containing 2 mM MgCl$_2$, 300 mM NaCl, 1% (v/v) Tween 20, and 10 mM imidazole). The cells were then disrupted by sonication, and the solution was centrifuged. The supernatant was loaded onto a TSKgel G3000SWXL column (Tosoh) equilibrated with Buffer A, linked to the BioAssist eZ system. The column was washed with Buffer B (50 mM sodium phosphate buffer (pH 8.0) containing 2 mM MgCl$_2$, 300 mM NaCl, 0.1% (v/v) Tween 20, 10% (v/v) glycerol, and 50 mM imidazole). The enzymes were then eluted with Buffer C (pH 8.0; Buffer B containing 250 mM imidazole instead of 50 mM imidazole). For purification of PaLhpC and PpLhpG, a buffer system without Tween 20 was used. PpLhpB and PpLhpC were stored at 4 °C and used within 1 day in further experiments, whereas PaLhpBFE and PpLhpG were concentrated by ultrafiltration with Centriplus YM-30 (Millipore), dialyzed against 50 mM Tris-HCl buffer (pH 8.0; Buffer B containing 250 mM imidazole instead of 50 mM imidazole), and stored at −35 °C until use.

The native molecular mass of recombinant proteins was estimated by gel filtration, which was carried out using an HPLC system at a flow rate of 1 ml/min. The purified enzyme was loaded onto a TSKgel G3000SWXL column (Tosoh) equilibrated with 50 mM Tris-HCl buffer (pH 9.0) containing 2 mM MgCl$_2$ and 50% (v/v) glycerol, and stored at −35 °C until use.

All enzyme assays were performed at 30 °C.

**D-HypDH Assay**—D-HypDH activity was assayed by monitoring the reduction rate of p-iodonitrotetrazolium violet (INT) (artificial electron acceptor) and phenazine methosulfate (PMS) (electron transfer intermediate) using a Shimadzu UV-1800 spectrophotometer (Shimadzu GLC Ltd., Tokyo, Japan). The standard reaction mixture contained 0.25 mM INT and 0.06 mM PMS in 50 mM Tris-HCl buffer (pH 9.0). The reaction was started by the addition of 100 mM D-hydroxyproline (100 µl) with a final reaction volume of 1 ml. The millimolar absorption coefficient (ε) for INT was 15.0 mM$^{-1}$cm$^{-1}$ at 490 nm. To estimate the specificity of electron acceptors, nitroblue tetrazolium (NBT) together with PMS (ε = 36.0 mM$^{-1}$cm$^{-1}$ at 530 nm), 2,6-dichloroindophenol (ε = 21.5 mM$^{-1}$cm$^{-1}$ at 600 nm), ferricyanide (ε = 1.04 mM$^{-1}$cm$^{-1}$ at 405 nm), horse liver cytochrome c (ε = 15.3 mM$^{-1}$cm$^{-1}$ at 553 nm), and NAD(P)$^+$ (ε = 6.2 mM$^{-1}$cm$^{-1}$ at 340 nm) at a final concentration of 0.25 mM were used. The kinetic parameters, $K_m$ and $k_{cat}$ values, were calculated by a Lineweaver-Burk plot.

**Pyr4H2C Deaminase Assay**—Pyr4H2C deaminase was assayed spectrophotometrically in the coupling system with KGSADH. The recombinant AbLhpG protein (KGSADH-III) was prepared by the method described previously (21). The reaction mixture (1 ml) consisted of 50 mM Tris-HCl buffer (pH 8.0), 10 mM Pyr4H2C, 1.5 mM NADP$^+$, and 1 unit of purified AbLhpG. Pyr4H2C was enzymatically synthesized from D-hydroxyproline by Pa$\alpha$/D-HypDH as described below. No change in absorbance at 340 nm was found at this stage. The reaction was started by the addition of a small amount of protein, and the increasing absorption caused by NADPH produced in the reaction was measured at 340 nm.

**KGSADH Assay**—KGSADH activity was assayed by the method described previously (20, 21).

**Zymogram Staining Analysis**

Cell-free extract or purified D-HypDH was separated by non-denaturing PAGE with a 10% (w/v) gel at 4 °C. The gels were then soaked in 10 ml of staining solution consisting of 50 mM Tris-HCl (pH 9.0), 2 mM MgCl$_2$, 0.25 mM INT, 0.06 mM PMS, and 10 mM D-hydroxyproline at room temperature for 15 min in the dark. Dehydrogenase activity appeared as a red band.

**Internal Peptide Analysis of Recombinant Pa$\alpha$/D-HypDH**

As described under “Results,” when the PaLhpBFE gene cluster was expressed in _P. putida_ cells, the purified enzyme consisted of three bands with different molecular masses in SDS-PAGE analysis (see Fig. 2G, lane 4). To identify these proteins with molecular masses of 39, 8, and 44 kDa as PaLhpB, PaLhpF and PaLhpE, respectively, each protein band on the 12% (w/v) SDS-PAGE gel was excised, and the gel pieces were incubated with 10 mM dithiothreitol at 56 °C for 45 min. Then protein in the gel was incubated with 55 mM iodoacetamide at room temperature for 10 min and subjected to in-gel digestion with trypsin (Promega). The digests were applied to a liquid chromatography (LC)-electrospray ionization-tandem mass spectrometer (LCQ Fleet™ LC-ESI-MSn system, ThermoFisher Scientific) in the positive ion mode. The data were collected in the data-dependent tandem mass spectrometry (MS/MS) scan mode and analyzed using SEQUEST software (ThermoFisher Scientific).

**Determination of Flavin Cofactor in D-HypDHs**

Purified Pp$\alpha$/D-HypDH and Pa$\alpha$/D-HypDH were dialyzed against H$_2$O, and cofactors were released by heat denaturation. After removal of the precipitate formed by centrifugation, the supernatant was used to identify the flavin cofactor(s) by HPLC. HPLC analysis was carried out using a TSKgel ODS-80TM column (4.6 × 150 mm; Tosoh). An isocratic elution (10 min) with 10 mM potassium phosphate (pH 6.0) followed by a linear gradient (30 min) between 0 and 70% methanol in the same solution was used for the elution. The flow rate was 1.0 ml/min, and
elution was monitored by the absorbance at 260 nm. Non-heme iron contents in Pa/D-HypDH were estimated by the method of Fish (29).

**Identification of Reaction Product of d-HypDHs**

The reaction mixture (200 ml) consisted of 50 mM Tris-HCl buffer (pH 9.0), 2 mM MgCl₂, 50 mM d-hydroxyproline, and 0.05 mM PMS. After the addition of 50 mg of purified Pp/d-HypDH or Pa/d-HypDH, the mixture was left at 30 °C in the dark overnight. Remaining PMS was removed by adding charcoal and filtered (referred to as Solution X). Solution X was applied to a Dowex 1X2 Cl⁻ form (100–200 mesh) resin column followed by a 0–1 M gradient of HCl. To identify a putative Δ¹-proline product, 125 μl of each fraction was added to 500 μl of 5% (w/v) p-dimethylaminobenzaldehyde in n-propanol followed by 1.25 ml of 3 N H₂SO₄. The mixtures were shaken thoroughly and incubated at 70 °C for 5 min, and the absorbance was measured at 550 nm. Active fractions were combined, adjusted to ~pH 11 with NaOH, and lyophilized. The ¹H NMR spectrum of d-hydroxyproline (400 MHz, D₂O) was as follows: 4.41 (1H, m), 4.04 (1H, dd, J₁ = 10 Hz, J₂ = 4 Hz), 3.30 (1H, dt, J₁ = 12 Hz, J₂ = 2 Hz), 3.20 (1H, dd, J₁ = 12 Hz, J₂ = 4 Hz), 2.34 (1H, m), and 2.09 (1H, m). As described under “Results,” Pyr4H₂C was not purified by this procedure. However, when Solution X was used as the substrate of d-HypDH, no significant activity was found, indicating the complete conversion of d-hydroxyproline to the reaction product. Therefore, this sample was used as 50 mM Pyr4H₂C solution for measuring the activity of PpLhpC.

**Target Disruption of L-Hydroxyproline Gene Cluster**

The Km' cassette of pUC4K was inserted into the single Sall site in the coding sequence of the PpLhpC gene of pQE/PpLhpC to yield pQE/PpLhpC::Km. To introduce the restriction site for BamHI at the 5' - and 3'-ends of the DNA fragment containing the Km' gene in the PpLhpC gene, PCR was carried out using pQE/PpLhpC::Km as a template and two primers, P7 and P8 (supplemental Table S1). The 2.3-kbp BamHI DNA fragment was subcloned into the BamHI site of the suicide vector pKNG101 (bacB Sm') (30) to yield pKNG/PpLhpC::Km (see Fig. 6C). This plasmid was introduced into parent strain P. putida KT2442, and the resulting transformants were selected for integration of the Km' marker in the chromosome on an LB agar plate containing 50 mg/liter kanamycin. The mutant was analyzed by colony PCR with P15 and P16 primers designed to target the flanking region of the PpLhpC gene to confirm the presence of its disrupted copy of the PpLhpC gene.

**Amino Acid Sequence Alignment and Phylogenetic Analysis**

Protein sequences were analyzed using Protein BLAST and the ClustalW program distributed by DDBJ (DNA Data Bank of Japan). The phylogenetic tree was produced using the TreeView 1.6.1 program.

**RESULTS**

**Gene Cluster Related to Bacterial L-Hydroxyproline Metabolism**—As described in the Introduction, we previously identified L-hydroxyproline-inducible KGSADH-III (referred to as LhpG in this report) from A. brasilense (18) (Fig. 2A). Protein BLAST analysis revealed that the homologous gene exists as a gene cluster on the genomes of many bacteria, including P. putida and P. aeruginosa, with the ability to grow on L-hydroxyproline. The gene cluster of P. putida consisted of five hypothetical genes, LhpB (FAD-dependent oxidoreductase), LhpG (KGSADH), LhpC (dihydrolipicolinate synthase), LhpA (epimerase), and LhpP (amino-acid permease) among which LhpB was a likely candidate for d-HypDH (Fig. 2A). Surprisingly, the gene cluster of P. aeruginosa contained several additional genes, LhpB (glycine/d-amino-acid oxidase), LhpE (heterotrameric sarcosine oxidase α-subunit), LhpF (ferrredoxin), and LhpD (malate/lactate dehydrogenase), in the order of LhpB-F-E-D (PaLhpF was not identified in the database, but we found an AbLhpF gene homolog between PaLhpB and PaLhpE genes). The 3'-part of PaLhpB and PaLhpF was slightly overlapped by the 5'-part of PaLhpF and PaLhpE, respectively (Fig. 2F, inset). A similar tendency was also found in heteromeric Tp/B-PDH (8) (Fig. 2B). This suggested the possibility that d-HypDHs of P. putida and P. aeruginosa are largely different and that PaLhpB-F-E encode each subunit in the heteromeric structure.

**Expression of Recombinant Proteins in P. putida Cells**—Although we first attempted to express PpLhpB, PaLhpB, PaLhpF, and PaLhpE proteins in E. coli cells, all mainly appeared in the fraction of the insoluble form despite efforts involving a survey of E. coli strains and vectors (data not shown). No improvement was found in the co-expression of PaLhpB-F-E. We previously revealed that P. putida KT2442-oxyR1 strain constitutively produces a soluble AhpC protein, a small subunit of alkyl hydroperoxide reductase, with a molecular mass of 24 kDa (Fig. 2G, lanes 1 and 2) (28). Therefore, we expressed His₆-tagged PpLhpB protein under regulation of the ahpC promoter inserted into the Escherichia-Pseudomonas shuttle plasmid in P. putida KT2442-oxyR1 cells (Fig. 2F) and purified it to homogeneity in a single step using immobilized metal (Ni²⁺) affinity chromatography. SDS-PAGE gave a single band with a molecular mass of ~47 kDa, which corresponded to the theoretical value of PpLhpB (46,856.49 Da) (Fig. 2G, lane 3). On the other hand, it was impossible to determine the native molecular mass because of the elution in the void volume of gel filtration column (Fig. 2H and supplemental Fig. S1). The purified enzyme was yellow, which is typical of flavoproteins, and showed 6.41 units/mg of protein specific activity toward d-hydroxyproline using standard assay conditions in the artificial electron acceptor system (PMS-INT; Table 1), indicating the function of d-hydroxyproline dehydrogenase (Pp/d-HypDH).

PpLhpB-F-E operon in which the His₆ tag sequence was attached at the N terminus of the LhpB gene was also expressed in ahpC promoter in P. putida KT2424-oxyR1 cells (Fig. 2F), and the recombinant protein was successfully purified to homogeneity. Native PAGE gave a single band (see Fig. 3B, inset), and a symmetric peak appeared in gel filtration analysis (supplemental Fig. S1). On SDS-PAGE analysis, two major distinct bands (referred to as α- and β-subunits, respectively) along with one minor band (referred to as γ-subunit) were observed (Fig. 2G, lane 4). The estimated molecular masses of α-, β-, and γ-subunits (48, 42, and 10 kDa, respectively) were similar to the theoretical values of PaLhpE, PaLhpB, and PaLhpF (44,055.06, 41,133.39, and 8,184.55 Da, respectively).
Furthermore, LC-electrospray ionization-MS/MS profiles using tryptic peptides of each protein band revealed that α-, β-, and γ-subunits corresponded to PaLhpE, PaLhpB, and PaLhpF, respectively (supplemental Fig. S2 and supplemental Tables S2–S4). Because the His6 tag sequence was attached only to the N terminus of PaLhpB, co-purification using Ni²⁺ affinity chro-
matography suggested that this protein consists of three different subunits. The molar ratio of \( \alpha: \beta: \gamma \) was \( \sim 1:1:1 \) as judged by image scanning of the SDS-PAGE peak areas using NIH ImageJ Version 1.45s software. The native molecular mass estimated as described in preliminary reports (13, 15).

TABLE 1

| Substrates   | Electron acceptors | Specific activity* \( \text{units/mg protein} \) | \( K_m \) \( \text{mM} \) | \( k_{\text{cat}} \) \( \text{min}^{-1} \) | \( k_{\text{cat}}/K_m \) \( \text{min}^{-1} \text{mM}^{-1} \) |
|--------------|--------------------|-----------------------------------------------|----------------|----------------|----------------|
| PpLhpB       | D-Hydroxyproline   | 6.41 ± 0.30                                  | 0.276 ± 0.012\(^b\) | 280 ± 11\(^b\) | 1.010 ± 4 |
|              | D-Proline          | 0.463 ± 0.03                                 | 0.0802 ± 0.008\(^b\) | 19.7 ± 1.3\(^c\) | 246 ± 9 |
|              | D-Hydroxyproline   | 1.28 ± 0.20                                  | 6.58 ± 1.15\(^d\) | 59.9 ± 7.5\(^d\) | 9.15 ± 0.45 |
|              | D-Proline          | 0.185 ± 0.002                                | 3.49 ± 0.34\(^d\) | 9.95 ± 0.66\(^d\) | 2.86 ± 0.09 |
| PaLhpBFE     | D-Hydroxyproline   | 21.1 ± 0.70                                  | 0.109 ± 0.009\(^f\) | 2230 ± 122\(^f\) | 20,500 ± 530 |
|              | D-Proline          | 5.66 ± 0.40                                  | 0.121 ± 0.002\(^f\) | 871 ± 10\(^f\) | 7,200 ± 50 |
|              | D-Hydroxyproline   | 7.05 ± 0.02                                  | 8.55 ± 0.35\(^f\) | 924 ± 28\(^f\) | 108 ± 1 |
|              | D-Proline          | 2.80 ± 0.05                                  | 11.0 ± 0.3\(^f\) | 507 ± 18\(^f\) | 46.0 ± 0.3 |

\(^a\) Under standard assay conditions as described under "Materials and Methods."  
\(^b\) Seven different concentrations of D-hydroxyproline between 0.01 and 0.1 mM were used.  
\(^c\) Six or seven different concentrations of D-proline between 1 and 6 mM were used.  
\(^d\) Nine different concentrations of D-hydroxyproline between 0.02 and 1 mM were used.  
\(^e\) Eight or 10 different concentrations of D-proline between 0.5 and 10 mM were used.  
\(^f\) Nine different concentrations of D-proline between 0.05 and 1010 mM were used.

Among the characterized amino-acid dehydrogenases, only D-PDH from a hyperthermophilic archaeon, *Pyrobaculum islandicum* (31), showed significant activity with D-hydroxyproline and close \( K_m \) values between D-proline and D-hydroxyproline (4.2 and 9.5 mM, respectively) (supplemental Table S6). This enzyme has broad substrate specificity with many D-amino acids in addition to D-hydroxyproline. Furthermore, 2,6-dichloroindophenol and/or ferricyanide is the most preferred electron acceptor(s) for known DL-PDHs, including *P. islandicum* D-PDH. To our knowledge, this is the first report of amino-acid dehydrogenase(s) with high specificity for D-hydroxyproline.

Effect of pH on Activity of D-HypDHs—pH activity profiles of Pp/D-HypDH and Pa/D-HypDH were similar: their maximal activities were observed at the range of pH 9 – 9.75 (supplemental Fig. S2). Furthermore, the enzymes maintained over 70% of their maximal activity after 2 h of incubation at pH 8 – 10.5 (data not shown). Apparent \( K_m \) values determined from the acidic limb of the profiles were 8.5 – 8.9. It is likely that these properties are similar to eukaryotic D-amino-acid oxidases (32) rather than archaeal DL-PDHs (supplemental Fig. S3).

Prosthetic Groups in D-HypDHs—As described above, both Pp/D-HypDH and Pa/D-HypDH were yellow (or orange-brown), and the addition of FAD or FMN in the standard assay mixture had no effect on the activity (data not shown). The UV-visible absorption spectrum of Pp/D-HypDH exhibited two pronounced absorption peaks at 354 – 360 and 454 – 456 nm, indicating typical flavoproteins (Fig. 3C). On the other hand, that of Pa/D-HypDH revealed absorption maxima at 353, 409, and 449 – 453 nm. Because the presence of [2Fe-2S] iron-sulfur cluster motifs was predictable on \( \alpha: \beta: \gamma \) subunits on the primary structure (see below), this spectrum is likely consistent with the sum of the spectra of typical flavoproteins (maxima at about 370 and 450 nm) and an oxidized [2Fe-2S] center (maxima at about 320, 415, and 455 nm): the latter was particularly similar to that of [2Fe-2S] ferredoxin from *E. coli* (cf. Fig. 5 in Ref. 33).

We attempted to identify the prosthetic cofactors in Pp/D-HypDH and Pa/D-HypDH using HPLC (Fig. 3D). Authentic FAD, FMN, ATP, and ADP were used as standards. As a result,
Pp/D-HypDH as well as most of the other amino-acid dehydrogenases contained only FAD. Although the molar value per mole of enzyme was unclear because the native molecular mass was not determined by gel filtration (supplemental Fig. S1), 0.02 mol of FAD was released from 1 mg of the purified enzyme. Because one FAD-binding motif was predictable on the primary structure (see below), the quaternary structure may be a homodimer. Interestingly, both FAD and FMN were detected within Pa/D-HypDH. Moreover, we determined that there were 8 mol of FAD and 4 mol of FMN/mol of enzyme.

On the other hand, iron contents per mole of enzyme were 7.8. Because the quaternary structure is αβγ, two FADs, one FMN, and one [2Fe-2S]-iron sulfur cluster might be contained within the αβγ heterotrimer.

Identification of Reaction Product by D-HypDH—The reaction products of D-hydroxyproline dehydrogenation catalyzed by Pp/D-HypDH and Pa/D-HypDH were purified using anion exchange chromatography and identified by NMR. Upper panels, authentic D-hydroxyproline; middle panels, reaction product; right panels, authentic pyrrole-2-carboxylate. Left panels, 1H NMR spectra showing the assignments of protons in D2O; right panels, 13C NMR spectra showing the assignments of carbons in D2O. Asterisks indicate peaks derived from an internal standard. In this analysis, pyrrole-2-carboxylate was identified as the reaction product (see in text). Similar results were obtained when Pp/D-HypDH was used as an alternative (data not shown). mAU, milliabsorbance units.

FIGURE 3. Characterization of Pp/D-HypDH and Pa/D-HypDH. Lineweaver-Burk plots of Pp/D-HypDH (A) and Pa/D-HypDH (B) toward D-hydroxyproline and D-proline using PMS-INT (circle) and PMS-NBT (square) assay systems, respectively, are shown. The inset shows zymogram staining analysis using L-proline (lane 1), D-proline (lane 2), L-hydroxyproline (lane 3), and D-hydroxyproline (lane 4) as substrates together with the PMS-INT assay system. Purified enzymes (10 μg) were applied. C, absorption spectra of Pp/D-HypDH (gray line) and Pa/D-HypDH (black line). Each enzyme solution used was 5 mg/ml. D, HPLC analysis of prosthetic groups. Elution profiles of the standard mixture (top) and extracts of Pp/D-HypDH (middle) and Pa/D-HypDH (bottom) are shown. E, identification of reaction product of Pa/D-HypDH by NMR. Upper panels, authentic D-hydroxyproline; middle panels, reaction product; right panels, authentic pyrrole-2-carboxylate.
D$_2$O, the NMR spectra overlapped completely (data not shown). It is known that in rats l-hydroxyproline is converted to Pyr4H2C by l-amino-acid oxidase (dehydrogenase) followed by pyrrole-2-carboxylate upon acidification of the reaction mixture via the unstable oxidation intermediate, probably Pyr4H2C (34). In this study, we observed that a pyrrole-related compound detected using p-dimethylaminobenzaldehyde is eluted from a column resin in the range of 0.7–1 M HCl binding motif with a proteins. This region contains a highly conserved typical ADP-(Fig. 2G, lane 5). The apparent molecular mass estimated by SDS-PAGE was 35 kDa, coinciding with the theoretical value of 35,341.67 Da. The native molecular mass estimated by gel filtration was ~275 kDa (Fig. 2H and supplemental Fig. S1), indicating a homooctameric structure.

As described above, LhpC belongs to the dihydrodipicolinate synthase/N-acetylneuraminic lyase family (22). Because most members of this protein family produce pyruvate and several aldehydes in the aldol cleavage reaction (see Fig. 5C), potential aldolation activity with Pyr4H2C in PpLhpC was first measured using lactic dehydrogenase as a coupling enzyme but was not detected (data not shown). Therefore, we alternatively used the purified recombinant AbLhpG (KGSADH), which shows high specificity with aKGSa, as a coupling enzyme (21) (also see next section). Under this assay system, PpLhpC showed specific activity of 2.41 units/mg of protein in the presence of 10 mM Pyr4H2C (and 1.5 mM NADP$^-$). Furthermore, when NADP$^+$-dependent l-glutamate dehydrogenase co-existed in the reaction mixture, the increase in absorbance at 340 nm was completely eliminated (data not shown) as a result of cooperative reactions by PpLhpC (Pyr4H2C $\rightarrow$ aKGSa + NH$_3$), KGSADH (aKGSa + NADP$^+$ $\rightarrow$ l-glutamate dehydrogenase) and l-glutamate dehydrogenase (a-ketoglutarate + NH$_3$ + NADP$^+$ $\rightarrow$ l-glutamate + NADP$^+$). These results suggested that PpLhpC plays a role as a Pyr4H2C deaminase. The $K_a$ and $k_{cat}$ values with Pyr4H2C were estimated to be 0.693 ± 0.031 mM and 71.5 ± 2.1 min$^{-1}$, respectively. Pyruvate is a common substrate for dihydrodipicolinate synthase/N-acetylneuraminic lyase proteins as described above. On the other hand, l-2-keto-3-deoxyarabinonate (l-KDA) has a hydroxyl group at the C5 position of Pyr4H2C (4-hydroxy-2-oxo-5-aminovalerate) instead of an amino group. Because l-KDA dehydratase also belongs to the dihydrodipicolinate synthase/N-acetylneuraminic lyase family (see “Discussion”), one possibility was that l-KDA is an alternative substrate for potential dehydration of Pyr4H2C deaminase. However, both pyruvate and l-KDA inhibited the enzyme competitively, and the $K_a$ values were 0.492 and 0.592 mM, respectively (Fig. 5A).

Functional Characterization of PpLhpG as KGSADH—In addition to PpLhpC, His$_x$-tagged PpLhpG was overexpressed in E. coli cells and purified to homogeneity with a nickel-chelating affinity column (Fig. 2G, lane 6). The apparent molecular mass of His$_x$-tagged PpLhpG was estimated by SDS-PAGE analysis as 55 kDa, coinciding with the theoretical value of 56,244.13 Da. The native molecular mass estimated by gel filtration was ~113 kDa (Fig. 2H and supplemental Fig. S1), indicating a homodimeric structure. As expected from the significant sequence similarity to AbLhpG (66% identity), the recombinant PpLhpG protein showed specific activities for aKGSa of 55.5 and 25.2

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**Novel Bacterial L-Hydroxyproline Pathway**

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**Functional Characterization of PpLhpC as Pyr4H2C Deaminase—**The LhpC gene is commonly contained within l-hydroxyproline operons of P. putida and P. aeruginosa (sequence identity of ~30%) (Fig. 2A). To estimate enzyme function, PpLhpC was overexpressed in E. coli cells by induction with isopropyl β-D-thiogalactopyranoside as a His$_x$-tagged protein and purified to homogeneity with a nickel-chelating affinity column (Fig. 2G, lane 5). The apparent molecular mass estimated by SDS-PAGE was 35 kDa, coinciding with the theoretical value of 35,341.67 Da. The native molecular mass estimated by gel filtration was ~275 kDa (Fig. 2H and supplemental Fig. S1), indicating a homooctameric structure.
FIGURE 4. Partial multiple sequence alignment of deduced amino acid sequences of D-HypDHs. GenBank accession numbers are shown in Fig. 2, A–E. Gray-shaded letters indicate highly conserved amino acid residues. Secondary structures of Ph/L-PDH1 (Protein Data Bank code 1Y56) are shown under the sequence: H9251 - helices, gray cylinders; H9252 - strands, gray arrows. A, alignment of Pp/D-HypDH, Hcn (PfHcnC), Nox (AtNoxB), Oox (AtOoxB), Ph/L-PDH1, Ph/L-PDH2, and Tp/l-PDH. The ADP-binding motif and FMN-binding sites (also in C) are shaded in red and green, respectively. B, alignment of Hcn (PfHcnA), Nox (AtNoxC), Oox (AtOoxC), and N-terminal regions of H9251 -subunits of Ph/L-PDH1, Ph/L-PDH2, and Tp/l-PDH. The [2Fe-2S]-binding motif is shaded in yellow. C, alignment of H9251 -subunits of Ph/L-PDH1, Ph/L-PDH2, and Tp/l-PDH and δ-subunits of Ph/l-PDH2 and Tp/l-PDH. Two ADP-binding motifs and the [2Fe-2S]-binding motif are shaded in orange, pink, and cyan, respectively. D, schematic subunit assembly of dehydrogenases. The colors of putative binding sites for prosthetic groups correspond to those in A–C. Figures on the right indicate the gene cluster encoding these proteins on the genomes of microorganisms. The deduced amino acid sequences with asterisks are shown in supplemental Table S3. E, structural formulas for D-hydroxyproline, glycine, D-nopaline, and D-octopine and schematic reactions by D-HypDH, Hcn, Nox, and Oox. The common moiety with D-hydroxyproline is shown in bold.
Novel Bacterial L-Hydroxyproline Pathway

**A**

![Graph showing enzyme activities with L-Hydroxyproline](image)

**B**

| Enzyme          | Reaction                        |
|-----------------|---------------------------------|
| AbLhpC          | 135 FMVVFVNF 162 NIVCRFDSSG     |
| PaLhpC          | 134 FMVVFVNF 161 NIVCRFDSSG     |
| PpLhpC          | 132 BAVTVSGF 159 NIVCRFDSSG     |
| ACH9801 (2HMC)  | 132 BAVTVSGF 159 NIVCRFDSSG     |
| AbArA (3FKK)    | 139 RMVODAD 166 QAYAIEETP       |
| FpRSDGH (AA2A5869) | 137 CVVVRVR 162 NIVCRFDSSG     |
| hHCOG (35SN)    | 146 FVVLWSP 191 NIVCRFDSSG     |
| SsRKGDA (1W3T)  | 126 FVVLWSP 150 CPTKQVPTTIE     |
| TrLgal (ABB04235) | 141 ELLVYYP 169 NIVCRFDSSG     |
| PeLR4 (ABB68603) | 138 FVVLWSP 166 NIVCRFDSSG     |
| NsCBPHA (BA2A3263) | 146 FVVLWSP 173 QINASHLGY     |
| PpHBFPH (AAA66357) | 151 AIALRRP 179 QVTFATYLG      |
| EcDHPS (1DHP)   | 129 EQLVYYP 156 NIVCRFDSSG     |

**C**

![Chemical structures and reactions](image)

**D**

![Pathway diagram](image)
Novel Bacterial L-Hydroxyproline Pathway

TABLE 2
Substrate specificity of KGSADH from P. putida

| Substrate         | Coenzyme | Specific activitya |
|-------------------|----------|-------------------|
|                   |          | PpLhpG | AbLhpG* |
| αKGSα             | NADP+    | 55.5 (100) | 16.4 (53.4) |
|                   | NAD      | 25.2 (45.4) | 30.7 (100) |
| Propionaldehyde (C3) | NADP+    | 0.045 (0.08) | 0.7 (2.3) |
| Butyaldehyde (C4)  | NADP+    | 2.5 (4.5) | 2.8 (9.0) |
| Valeraldehyde (C5) | NADP+    | 2.9 (5.2) | 2.6 (8.3) |
| Hexylaldehyde (C6) | NADP+    | 4.5 (8.1) | 6.8 (22.2) |
| Octylaldehyde (C8) | NADP+    | 1.1 (2.0) | 6.4 (20.8) |
|                   | NAD      | 0.98 (1.8) | 3.8 (12.3) |

units/mg protein of protein in the presence of NADP+ and NAD+, respectively (Table 2). The kcat/Km with NADP+ (83,500 min⁻¹·μM⁻¹) was 134-fold higher than that with NAD+, mainly caused by the lower Km values (0.0230 and 6.15 mM, respectively; Table 3). Under the same assay conditions, AbLhpG showed similar kcat/Km values to αKGSα in the presence of NAD+ or NADP+, whereas kcat/Km with NADP+ was ~22-fold higher than with NAD+ (21). Various aldehydes, including αKGSα, were tested as substrates for dehydrogenation by PpLhpG in the presence of NADP+. αKGSα was clearly the best substrate, whereas activities with a number of aliphatic aldehydes from C3 to C8 were only 10% less than those with αKGSα (Table 2). These results indicated that PpLhpG possesses NADP+ preference and high specificity toward αKGSα similar to AbLhpG.

Gene Regulation and Disruptant Analysis—When P. putida and P. aeruginosa were grown on L-hydroxyproline or D-hydroxyproline, almost all four metabolic enzymes were induced in cell-free extracts (Fig. 6A). The induction of D-HypDH was also detected in zymogram staining analysis (Fig. 6B). Although (partially) incomplete induction of KGSADH activity was found in P. aeruginosa, it has been reported that this bacterium possesses a constitutively expressed KGSADH isozyme(s) and/or other aldehyde dehydrogenase(s) with activity for αKGSα (14). To estimate the physiological role(s) of the Lhp gene cluster in L-hydroxyproline metabolism, we carried out gene disruption experiments by introducing a Km′ gene at the center of the PpLhpC gene encoding Pyr4H2C deaminase (Fig. 6, C and D). The obtained LhpC− mutant strain was distinct from the wild-type strain (KT2442) in that neither L-hydroxyproline nor D-hydroxyproline was a sole carbon source supported growth (Fig. 6E). On the other hand, there was no difference in growth on D-proline (an alternative substrate for D-HypDH), glucose, and L-proline between the two strains. Indeed, only L-hydroxyproline 2-epimerase activity was significantly found in the cell-free extract prepared from LhpC− mutant cells grown on LB medium supplemented with L-hydroxyproline; D-HypDH, Pyr4H2C deaminase, and KGSADH activities were not found (data not shown). These results indicated that LhpC disruption also led to the inactivation of LhpB and LhpG genes within the Lhp operon (Fig. 2A). Overall, these results clearly suggested that the Lhp gene cluster is involved in L-hydroxyproline (as well as D-hydroxyproline) but not D-proline metabolism.

DISCUSSION

Comparisons between Pa/D-HypDH and Pp/D-HypDH—Although Pa/D-HypDH and Pp/D-HypDH show very high specificity for D-hydroxyproline (as a substrate) and PMS-INT or PMS-NBT (as an electron acceptor), the former heteromeric enzyme contains FAD, FMN, and a [2Fe-2S] iron-sulfur cluster as prosthetic groups, which is different from the latter homomeric enzyme (only FAD), strongly indicating that D-HypDHs appeared convergently in P. putida and P. aeruginosa during an evolutionary stage. Only P. islandicum D-PDH shows significant activity for D-hydroxyproline in addition to D-proline, and the manner of dehydrogenation reaction is homologous to that of D-HypDH (31). However, it is likely that their substrate specificities are also acquired independently of each other because their sequence similarities are not high.

In this study, we first revealed that Pa/D-HypDH is phylogenetically related to hydrogen cyanide synthase (Hcn), D-nopaline oxidase (Nox), and D-octopine oxidase (Oox) of bacteria (37–39). Hcn, Nox, and Oox complexes are encoded by hcnA-B-C, noxB-C-A, and ooxB-C-A gene clusters, respectively, on the bacterial genome (Fig. 2, C, D, and E). Furthermore, each substrate of Pa/D-HypDH, Hcn, Nox, and Oox possesses structural formulas similar to the secondary amine (Fig. 4E), clearly indicating that they evolved from a common ancestor. Although it is likely that Hcn, Nox, and Oox are membrane-associated flavoproteins as are most amino-acid dehydrogenases (31, 35), the detailed features have not been reported. Pa/D-HypDH is the first of this type of protein to be experimentally characterized at the molecular level.

Comparisons with D-PDHs from Archaea—D-PDHs, in particular Pa/D-HypDH, show high sequence similarities to several dehydrogenases for L- and D-proline from archaea.
Their schematic structures are illustrated in Fig. 4D. The β-subunits of the heteromeric enzymes correspond to homomeric enzymes and commonly function as catalytic subunits, which contain one ADP-binding motif in the N-terminal region (for FAD binding) (shaded in red). In fact, Tp/L-PDH shows dehydrogenation activity for L-proline by itself (8). On the other hand, the α-subunits may contribute to the regulation of catalysis because of the binding of several prosthetic group(s), including FAD (Pa/D-HypDH), ATP (Ph/L-PDH1α), and FAD and NADH (Tp/L-PDHα) at the ADP-binding motif(s) (shaded in orange and pink). The FMN molecule is located at the interface of α- and β-subunits in Ph/L-PDH1 (11), and the isoalloxazine ring is sandwiched by two hydrophobic residues, ßArg46 (shaded in green), suggesting a similar manner of FMN binding to Ph/L-PDH1α (10). When compared with FAD and FMN, the functional roles of the iron-sulfur cluster in catalysis are unclear. Cysteine-cluster ([2Fe-2S]-binding motif) in Ph/L-PDH1α (shaded in cyan) may be structurally conserved in Pa/D-HypDH and Tp/L-PDHβ, whereas the homologous [2Fe-2S]-binding motifs in Pa/D-HypDH and Tp/L-PDHα (shaded in yellow) are not found in Ph/L-PDH1α. Furthermore, only Tp/L-PDHβ contains the [8Fe-8S] cluster. This suggests that the components and structural location of iron-sulfur clusters in Pa/D-HypDH may be different from Ph/L-PDH1 and Tp/L-PDH.

We found that putative L-PDH or D-HypDH of microorganisms is classified into various types by bioinformatics analysis (Fig. 4D and supplemental Table S5). Interestingly, Pa/D-HypDH and Tp/L-PDHβ, which belong to “Types 3 and 2,”...
residues, respectively, correspond to the first ~100 and last ~70 amino acid residues, respectively, of Ph/L-PDH1 (Fig. 4A). Therefore, it is likely that these heteromeric proteins evolved from a common ancestral flavoenzyme (Type I), such as homomeric Pp/d-HydDH, by genetic duplication, division, and/or fusion and that they eventually acquired a new function(s).

**PyrH2C Deaminase within Aldolase Protein Family**—It is likely that PyrH2C deaminase belongs to the dihydrodipicolinate synthase/N-acetylneuraminic lyase family based on the phylogenetic analysis. Generally, members of this protein family possess a common (βα)8-barrel fold and share a common reaction step in their reactions, namely the formation of a “Schiff base” intermediate between a strictly conserved lysine residue and C2 carbon of the common α-keto acid moiety of the substrate (22) (Fig. 5B). The phenolic hydroxyl group of the tightly conserved tyrosine residue forms double hydrogen bonds to carboxyl and hydroxyl groups at position 4 of the substrate. Interestingly, 4-hydroxy-2-oxoglutarate aldolase, which is involved in mammalian l-hydroxyproline metabolism (Fig. 1A), is also a member of this protein family (4). Although lysine (Lys164) and tyrosine (Tyr136) align well with these residues in the sequence of PpLhpC (Fig. 5B), there is a poor phylogenetic relationship to any subfamilies of the other members (Fig. 5D), which is probably the reason for the unique enzyme catalysis (Fig. 5C). Site-directed mutagenesis and/or crystallographic analysis of PyrH2C deaminase would provide clearer evidence for assignment to the dihydrodipicolinate synthase/N-acetylneuraminic lyase protein family and help to elucidate novel catalytic mechanism in this protein family.

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