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Cloning and High-Level Expression of the Glutathione-Independent Formaldehyde Dehydrogenase Gene from *Pseudomonas putida*

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A DNA fragment of 485 bp was specifically amplified by PCR with primers based on the N-terminal sequence of the purified formaldehyde dehydrogenase (EC 1.2.1.46) from *Pseudomonas putida* and on that of a cyanogen bromide-derived peptide. With this product as a probe, a gene coding for formaldehyde dehydrogenase (*fdhA*) in *P. putida* chromosomal DNA was cloned in *Escherichia coli* DH5α. Sequencing analysis revealed that the *fdhA* gene contained 1,197-bp open reading frame, encoding a protein composed of 399 amino acid residues whose calculated molecular weight was 42,082. The transformant of *E. coli* DH5α harboring the hybrid plasmid, pFDHK3DN71, showed about 50-fold-higher formaldehyde dehydrogenase activity than *P. putida*. The predicted amino acid sequence contained several features characteristic of the zinc-containing medium-chain alcohol dehydrogenase (ADH) family. Most of the glycine residues strictly conserved within the family, including a Gly-Xaa-Gly-Xaa-Xaa-Gly pattern in the coenzyme binding domain, were well conserved in this enzyme. Regions around both the catalytic and the structural zinc atoms were also conserved. Analyses of structural and enzymatic characteristics indicated that *P. putida* FDH belongs to the medium-chain ADH family, with mixed properties of mammalian class I and III ADHs.

Formaldehyde dehydrogenase (FDH) (EC 1.2.1.1) has been reported to be present in a wide variety of organisms, from *Escherichia coli* to humans (37, 38). These enzymes are commonly NAD^+ linked and require glutathione to function. The reaction product of the enzyme was shown to be *S*-formylglutathione, and the true substrate was shown to be *S*-hydroxymethylglutathione, which is formed nonenzymatically from formaldehyde and glutathione. This rection is reversible (38). It was shown that human liver as well as several other sources also contain a highly active, specific enzyme, *S*-formylglutathione hydrolase, which catalyzes the irreversible hydrolysis of *S*-formylglutathione into formate and glutathione (38).

Koivusalo et al. (24) proved that FDH from rat liver was identical to the class III alcohol dehydrogenase (ADH), and later this was confirmed for the enzyme from human liver by Holmquist and Vallee (14). Class III ADH belongs to the medium-chain ADH family (18); members of this family from many sources have been extensively studied (17). The horse liver enzyme tertiary structure was characterized early by X-ray crystallography (10). For humans, this family has been categorized into at least three classes, I, II, and III (16). All classes contain enzymatically essential zinc and have clearly related structures. Among the family, class III ADH is the only enzyme that can catalyze the glutathione-dependent oxidation of formaldehyde. In addition, it has a very low affinity for ethanol but is capable of oxidizing long-chain aliphatic alcohols (41).

In 1979, Ando et al. found and purified an enzyme (FDH) (EC 1.2.1.46) from *Pseudomonas putida* C-83 which catalyzed irreversible oxidation of formaldehyde to formate without the external addition of glutathione (2). The enzyme required only an electron acceptor, NAD^+. Addition of glutathione did not affect the reaction rate. The activity was markedly inhibited by *p*-chloromercuribenzoate (PCMB). Ogushi et al. concluded that one of the PCMB-reactive sulfhydryl groups is essential for the binding of formaldehyde from the fact that formaldehyde protected one sulfhydryl group from the modification of the enzyme by PCMB (28).

P. putida FDH is a zinc-containing metalloenzyme which is inhibited by *o*-phenanthroline (27). At high pH values it can catalyze the NAD^+-dependent oxidation of long-chain alcohols such as *n*-pentanol, but it is inactive with ethanol. In these respects, *P. putida* FDH has characteristics similar to those observed for class III ADHs. It is of great interest to clarify whether *P. putida* FDH is structurally related to other glutathione-dependent FDHs. This paper deals with the cloning, sequencing, and expression of the *P. putida* FDH gene in *E. coli* and the comparison of the deduced amino acid sequence with those of zinc-containing medium-chain ADHs. The results revealed distant but clear structural relationships among them.

MATERIALS AND METHODS

Materials. A partially purified commercial preparation of *P. putida* FDH was kindly supplied by TOYOBO Co. Phenazine methosulfate, nitroblue tetrazolium, and 4-methylpyrazole were products of Nacalai Tesque. NAD^+ was from Kojin Kagaku Co. Restriction endonucleases, a Kilosequence deletion kit, AmpliTaq DNA polymerase, and various DNA-modifying enzymes were purchased from Takara Shuzo Co. The Sequenase version 2.0 DNA sequencing kit, *Csp*45I, and *Sac*II were from Toyobo Co. Deaza G/A Sequencing Mixes and [α-^{32}P]dCTP (3,000 Ci/mmol) were purchased from Pharmacia P-L Biochemicals Inc. and Amersham, respectively. *Staphylococcus aureus* V8 protease, lysozyme, RNase A, *Saccharomyces cerevisiae* ADH, and ATP were from Sigma, and calf intestine alkaline phosphatase was from Boehringer Mannheim. Oligonucleotides were synthesized by the phosphoramidite method with a Pharmacia LKB DNA synthesizer, Gene Assembler Plus.

Bacterial strains. *E. coli* DH5α [F$^-$ φ80d*lacZ*ΔM15

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Δ(*lacIZYA-argF*)U169 *recA1 endA1 gyrA96 thi-1 supE44 relA1 mcrA mcrB*$^+$ *hsdR17*] and JM105 [F′ (*traD36 proAB*$^+$ *lacI*^{q}Δ*lacZ*M15) Δ(*lac-proAB*) *thi endA1 supE sbcB15 rpsL hsdR4*] were used as the hosts for cloning. Plasmids pBR322 and pBluescript SK(−) and phagemids M13mp18 and M13mp19 were used for cloning. Bacteria were grown in Luria-Bertani broth.

Assay of FDH activity. Activity assays were performed by two different methods.

(i) Formation of diformazan. The reaction mixture (1.2 ml), containing 50 mM Tris-HCl (pH 8.0), 0.2% Triton X-100, 2 mM formaldehyde, 1 mM NAD^+, 25 μM phenazine methosulfate, 100 μM nitroblue tetrazolium, and 0.002 to 0.015 U of enzyme, was incubated for 15 min at 37°C. The reaction was stopped by the addition of 3 ml of 0.3 N HCl, and diformazan formed via the NADH-phenazine methosulfate-nitroblue tetrazolium coupling reaction was spectrophotometrically measured at 570 nm. One unit was defined as the amount of enzyme which formed 0.5 μmol of diformazan per min under the above-described conditions.

(ii) Formation of NADH. For the determination of kinetic parameters, activity was assayed by monitoring the formation of NADH at 340 nm with a reaction mixture of 50 mM glycine-NaOH (pH 8.9), 0.1 to 2 mM formaldehyde, 1 mM NAD^+, and 0.005 to 0.01 U of enzyme at 37°C.

Purification of the native enzyme. All purification procedures were carried out at 4°C unless otherwise stated. A commercial preparation of *P. putida* FDH (TOYOBO) (30 mg) was dissolved in 3 ml of 20 mM K-phosphate buffer (pH 6.8) and passed through a column (1.5 by 68 cm) of Hiload Superdex 200 pg (fast protein liquid chromatography system; Pharmacia) equilibrated with the same buffer at a flow rate of 0.5 ml/min. Three-milliliter fractions were collected. This step was repeated three times. Active fractions were combined and applied to a column (1.4 by 14 cm) of hydroxyapatite equilibrated with the same buffer at a flow rate of 0.15 ml/min. The adsorbed enzyme was eluted by using an increasing linear gradient of 20 to 500 mM K-phosphate buffer (pH 6.8) in a total volume of 300 ml, and 5-ml fractions were collected. Active fractions were combined and dialyzed against distilled water. The final preparation was lyophilized and stored at −20°C.

Proteolytic and CNBr cleavages of the purified FDH and amino acid sequence analyses of the peptide fragments derived. For proteolytic cleavage, the purified enzyme (1.2 mg) was digested with 60 μg of *S. aureus* V8 protease in 1 ml of 0.1 M K-phosphate buffer (pH 7.8) for 24 h at 37°C. For cyanogen bromide (CNBr) cleavage, the enzyme (1 mg) was incubated in 1 ml of 70% formic acid solution containing 2.2 mg of CNBr under N_2 gas in the dark for 15 h at room temperature, diluted with 3 ml of distilled water, and then lyophilized. Peptide mixtures obtained by enzymatic and CNBr cleavages were subjected to reversed-phase high-pressure liquid chromatography on a Vydac C18 column (particle size, 5 μm; pore diameter, 300 Å [30 nm]; 4.6 by 250 mm; Separations), using a solvent system of 0.075% trifluoroacetic acid in water versus acetonitrile–2-propanol (3:1) in 0.06% trifluoroacetic acid. Amino acid sequences of the amino-terminal region of the purified enzyme and of peptides derived from V8 protease and CNBr cleavages were determined by the manual Edman degradation method (23).

Preparation of DNAs. *P. putida* chromosomal DNA was prepared by the method of Saito and Miura (31). Plasmids were isolated by alkali lysis followed by polyethylene glycol 6000 precipitation. Single-stranded DNAs were prepared by a standard protocol (33) with JM105 as the host *E. coli* strain.

PCR. PCR was carried out in a 100 μl of a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 5% dimethyl sulfoxide, 0.01% gelatin, 0.2 mM each deoxynucleoside triphosphate, 1 μM each primer, 0.2 μg of chromosomal DNA from *P. putida*, and 2.5 U of AmpliTaq DNA polymerase. The sequences of the primers used were as follows: N terminal (N1), 5′-(A/C)A(A/T)CGTGGTGT(T/C/A/G)GT(T/C/A/G)TA(T/C)(T/C)T-3′; B7, 5′-A(A/G)(A/G)TCIC(G/T)IAT(T/C)TT(T/C)TCCAT-3′. Amplification consisted of 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, with a final extension at 72°C for 7 min. Exclusion of dimethyl sulfoxide gave minor, nonspecific amplification products (see Fig. 1). The PCR product was fractionated on a 1.2% low-melting-temperature agarose gel and visualized by ethidium bromide staining. It was purified by phenol extraction and precipitated with ethanol. After being blunted by Klenow fragment, the fragment was subcloned into the *Sma*I site of M13mp18 and sequenced by the chain termination method (34).

Labeling of DNA. DNA fragments were labeled by the method of Feinberg and Vogelstein (11), using a random primer DNA labeling kit (Takara Shuzo Co.).

Southern hybridization analysis. *P. putida* chromosomal DNA digested with various restriction enzymes was separated on a 1.0% agarose gel and transferred to a nitrocellulose filter. DNAs on the filter were fixed by heating for 2 h at 80°C. The filter was prehybridized for 2 h at 42°C in a solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM Na-phosphate buffer (pH 7.5), 1× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 100 μg of denatured salmon testis DNA per ml. Hybridization was carried out in 10 ml of the same solution containing a radiolabeled probe for 16 h at 42°C. After the incubation, the filter was washed twice in 100 ml of 2× SSC containing 0.1% SDS for 5 min at room temperature and twice in 100 ml of the same solution for 30 min at 60°C and then air dried. The filter was wrapped and exposed to an X-ray film with an intensifying screen at −80°C.

Preparation and screening of a library. *P. putida* chromosomal DNA was digested with *Eco*RI and size fractionated on a 1.0% agarose gel. DNA fragments were purified and ligated into the *Eco*RI site of pBR322 treated with calf intestine alkaline phosphatase. *E. coli* DH5α was transformed with the ligation mixture and plated on Luria-Bertani plates containing 100 μg of ampicillin per ml. Colonies of the transformants were transferred to nitrocellulose filters and then lysed. The liberated DNA was fixed, and hybridization was carried out as described above.

Subcloning and nucleotide sequence analyses. pBluescript SK(−) (Stratagene) was used for subcloning by standard protocols (33). The hybrid plasmid pFDHK3, which carried a 2.9-kb insert at the *Kpn*I site of pBluescript SK, was used to construct deletion mutants. pFDHK3 was digested with *Sac*I and *Eco*RI, and the linearized DNA was deleted from the *Eco*RI site on the vector by using a Kilosequence deletion kit (Takara Shuzo Co.).

Single-stranded DNAs were prepared from the series of deleted mutants described above by using R408 helper phage according to the manufacturer's instruction. Various restriction fragments were also generated from pFDHK3DN71 by using the restriction sites shown in Fig. 2 and were subcloned into both M13mp18 and M13mp19 directly or after being blunted. Nucleotide sequencing was carried out by the dideoxy chain termination method (34) with these single-stranded DNAs as templates. Both strands of all the fragments between

the deletion point of pFDHK3DN71 and the *Apa*I site were sequenced.

Purification of the enzyme expressed in *E. coli*. *E. coli* DH5α harboring pFDHK3DN71 was aerobically grown in 12 liters of N broth containing ampicillin (50 mg/liter) at 30°C for 18 h in a jar fermentor (New Brunswick). Harvested cells (about 30 g [wet weight]) were washed with 50 mM Tris-HCl buffer (pH 7.2) and resuspended in 300 ml of the same buffer. The cells were disrupted with glass beads in a Dyno-Mill, and the resultant suspension (about 700 ml) was centrifuged at 10,000 × *g* for 20 min to remove cell debris. To the supernatant was added 2% protamine sulfate dropwise to give 18 mg/g (wet weight) of cells in order to remove chromosomes and viscous materials. The mixture was kept for 30 min in an ice bath and centrifuged as described above. Solid ammonium sulfate was added to the supernatant to 45% saturation, and the mixture was kept for 30 min in an ice bath. After centrifugation at 15,000 × *g* for 20 min, the supernatant was fractionated by adding solid ammonium sulfate to 70% saturation. The mixture was kept for 1 h in an ice bath and centrifuged at 15,000 × *g* for 30 min. The precipitate was dissolved in 150 ml of 20 mM Tris-HCl buffer (pH 7.2) containing 40% saturated ammonium sulfate. The resultant solution was applied to a column (5 by 20 cm) of Toyopearl HW65C (32) equilibrated with this buffer, and 18-ml fractions were collected. After the column was washed with 500 ml of the same buffer, the adsorbed enzyme was eluted with a decreasing linear gradient of ammonium sulfate at a flow rate of 1.8 ml/min. The reservoir and mixing chamber contained 1 liter each of 0 and 40% saturated ammonium sulfate in 20 mM Tris-HCl buffer (pH 7.2). The enzyme was eluted at an ammonium sulfate concentration of 20% saturation. Active fractions were combined and precipitated by ammonium sulfate at 70% saturation for 30 min at 4°C. The precipitate was collected by centrifugation at 15,000 × *g* for 20 min, dissolved in 10 ml of 20 mM K-phosphate buffer (pH 6.8), and dialyzed against the same buffer. FDH was further purified to homogeneity by hydroxyapatite chromatography as described for the purification of the commercial enzyme, except that the gradient was made of 300 ml each of 20 and 500 mM K-phosphate (pH 6.8). Active fractions were combined, and the protein was precipitated by ammonium sulfate at 70% saturation. The precipitate was dissolved in 20 mM Tris-HCl (pH 7.2) and dialyzed against the same buffer. The final preparation was stored frozen in aliquots at −20°C.

Analytical methods. Protein concentrations were determined by the modified Lowry method with bovine serum albumin as a standard (30). The molecular weight of the enzyme was estimated by gel filtration on a Superdex 200 pg column calibrated with *E. coli* aminopeptidase P (200 kDa) (42), *S. cerevisiae* ADH (140 kDa), bovine serum albumin (67 kDa), and *Bacillus coagulans* proline iminopeptidase (32 kDa) (22) as markers. Gel filtration was carried out as described for the purification of the commercial enzyme, except that 0.5 ml of each sample (0.5 mg) was applied to the column. The elution volume of each protein was determined by measuring the A_{280}. Isoelectric focusing was performed with a commercially available gel (IEF 3-9 gel; Pharmacia), using the Pharmacia PhastSystem apparatus according to the manufacturer's instructions. To determine the isoelectric point, the pI calibration kit from Pharmacia was used as a standard.

K_m values were determined with Lineweaver-Burk plots by the NADH formation assay method. K_i values for 4-methylpyrazole were estimated as well, using inhibitor concentrations of 25 and 50 μM. Thermal and pH stabilities of FDH were determined by measuring the residual activities after the following treatments. For determination of thermal stability, the purified enzyme (0.1 U) was incubated in 0.1 ml of 100 mM Tris-HCl buffer (pH 8.0) for 30 min at various temperatures (0 to 80°C). For determination of pH stability, the purified enzyme (0.1 U) was incubated in 0.1 ml of various buffers (100 mM) for 30 min at 37°C; the buffers used were Na-acetate buffer (pH 4.0 to 5.5), K-phosphate buffer (pH 6.0 to 7.5), Tris-HCl buffer (pH 7.0 to 8.4), glycine-NaOH buffer (pH 8.4 to 11.5), and Na-bicarbonate–Na-carbonate buffer (pH 9.0 to 10.5).

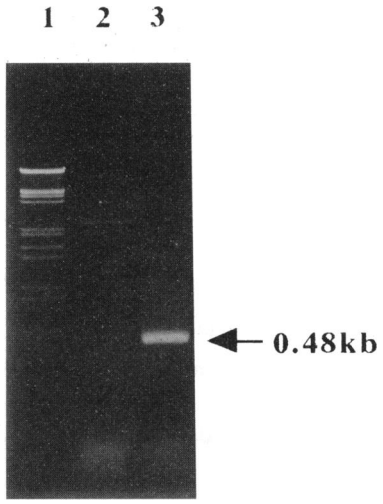

FIG. 1. Agarose gel electrophoresis of PCR products. Amplifications were performed in the presence (lane 3) or absence (lane 2) of 5% dimethyl sulfoxide as described in Materials and Methods. Lane 1, λ DNA digested with *Eco*RI and *Hin*dIII.

The DNA and protein sequences were analyzed by using the GENETYX-MAC genetic information processing software (Software Development Co., Ltd., Tokyo, Japan).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases with the accession number D21201.

RESULTS

Determination of partial amino acid sequence of *P. putida* FDH. To synthesize oligonucleotide primers for PCR, the N-terminal amino acid sequence of the purified enzyme and those of major peptide fragments derived by V8 protease or by CNBr cleavage were determined. As shown below all of these sequences coincided well with those deduced from the nucleotide sequence of the enzyme gene (see Fig. 5).

Amplification of FDH gene fragment. Upstream and downstream primers were designed from the N-terminal amino acid sequences of the native enzyme and of a CNBr-derived peptide, B7, respectively. A methionine residue was assumed to occur before the CNBr-derived peptide. Amplification of a *P. putida* DNA fragment mediated by PCR with primers N1 (upstream) and B7 (downstream) gave a single PCR product of approximately 0.48 kb in length (Fig. 1). This PCR product was subcloned into M13mp19, and its nucleotide sequence was determined. We concluded that the 485-bp fragment was a portion of the FDH gene (*fdhA*), since amino acid sequences of seven peptide fragments derived from the purified enzyme by proteolytic and CNBr cleavages were found in the amino

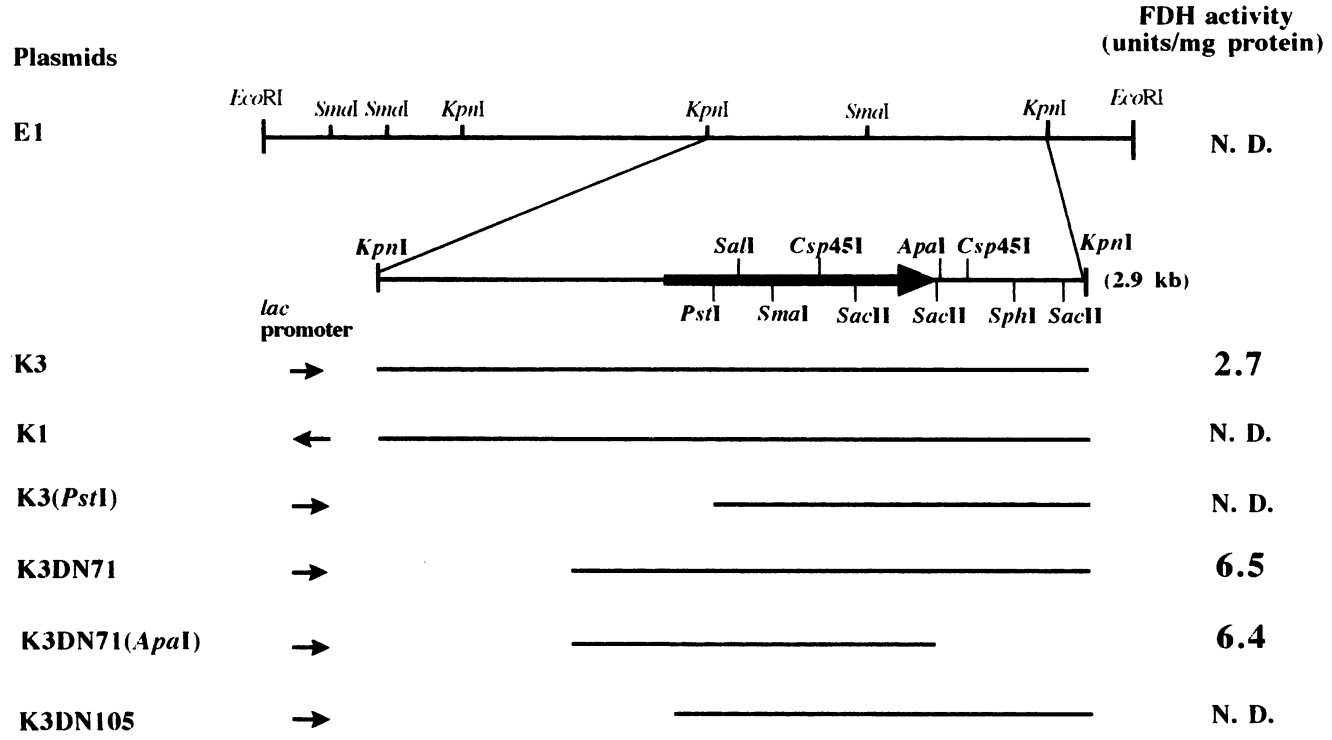

FIG. 2. Restriction map of the clone E1 and deletion analysis of the 2.9-kb *Kpn*I fragment. The lines indicate the inserted DNA fragments. FDH activities were assayed with sonic extracts prepared from the transformants containing various plasmids by the diformazan formation method described in Materials and Methods. The large arrow indicates the position and the direction of the enzyme gene. Orientations of the *lac* promoter relative to the inserted DNA are shown by small arrows. N.D., not detected.

acid sequence deduced from the nucleotide sequence of this PCR product (see Fig. 5).

Screening of *P. putida fdhA*. *P. putida* chromosomal DNA was digested with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Sal*I. The enzyme digests were fractionated by 1% agarose gel electrophoresis and transferred to nitrocellulose filters. Hybridization was carried out with the ^{32}P-labeled PCR product as a probe. An *Eco*RI fragment of 9 kb was found to hybridize to the probe. *Eco*RI fragments of around 9 kb were extracted from the agarose gel, ligated into the *Eco*RI site of pBR322, and used to transform *E. coli* DH5α. The resultant recombinant *E. coli* library containing *P. putida* chromosomal DNA fragments was screened by colony hybridization with the same probe. Eight of approximately 1,000 colonies hybridized to the probe. Restriction analysis revealed that all the positive clones had the same 9-kb insert in pBR322 at the *Eco*RI site. We selected one clone, named E1, for further experiments.

Subcloning and expression of *fdhA* in *E. coli*. No FDH activity was detected in *E. coli* DH5α transformed with E1, presumably because of the lack of an active promoter in *E. coli* within the insert on E1. To construct an expression plasmid, a restriction map for E1 was generated (Fig. 2). Southern hybridization analysis revealed that the structural gene for FDH existed within the 2.9-kb *Kpn*I fragment. This *Kpn*I fragment was subcloned into pBluescript SK. Two kinds of clones with respect to the orientation of the insert (K1 and K3) were obtained. The recombinant *E. coli* DH5α harboring K3 showed high-level FDH activity, whereas the one harboring K1 showed no FDH activity, indicating that the transcription of the enzyme gene was dependent on the *lac* promoter on the vector. To determine the approximate location of the enzyme gene in K3, a series of deleted mutants was constructed, and FDH activities in the respective transformants were measured. As shown in Fig. 2, K3 (*Pst*I), which has a *Pst*I-*Kpn*I fragment of the same orientation as in K3, showed no FDH activity. The 2.9-kb DNA insert in K3 was deleted from the upstream part of the gene. Deletion of 0.8 kb (DN71) increased the activity of the transformant by approximately twofold, but further deletions beyond 1.1 kb (DN105) resulted in loss of FDH activity. Removal of the *Apa*I-*Kpn*I fragment of the 3′ side of the gene from DN71 had little effect on the FDH activity. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the total protein contents of the transformants is shown in Fig. 3. Major bands with an apparent molecular weight of 42,000 were evident, and the amounts of the protein bands seemed to pararell the enzyme activities. These results suggested that the structural gene for FDH was located between the deletion point of DN71 and the *Apa*I site.

Purification and some properties of the expressed enzyme. Table 1 summarizes the purification of the expressed enzyme. The enzyme was purified to near homogeneity as verified by SDS-PAGE (Fig. 4), with a recovery of 45% of the activity from the cell extracts of *E. coli* DH5α harboring pFDHK3DN71. The N-terminal amino acid was found to be a serine, indicating that the methionine residue encoded by the initiation codon was similarly removed in both bacteria.

In Table 2, some properties of the expressed enzyme are compared with those of the native enzyme purified from *P. putida*. The two enzymes showed almost the same properties. The molecular weight was estimated to be 150,000 by gel filtration, while SDS-PAGE analysis gave a value of 42,000, suggesting that the enzyme exists as a tetrameric form. The isoelectric point calculated from the sequence data was 5.0, which agreed well both with the value previously estimated by

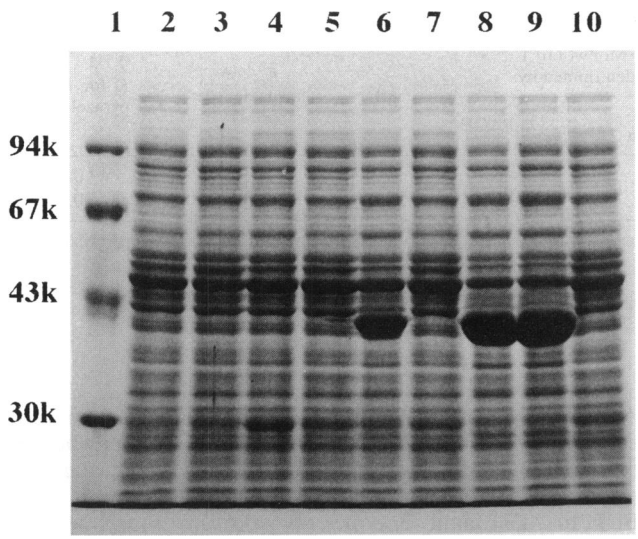

FIG. 3. SDS-PAGE of cell extracts prepared from *E. coli* DH5α harboring various plasmids. Cell extracts (about 20 μg) and the purified enzymes (3 μg) were electrophoresed in a 10% polyacrylamide gel and stained with Coomassie brilliant blue R-250 by the method of Laemmli (25). Lane 1, marker proteins (94,000 molecular weight [94k], phosphorylase *b*; 67k, bovine serum albumin; 43k, ovalbumin; 30k, carbonic anhydrase); lane 2, *E. coli* DH5α; lane 3, *E. coli* DH5α/pBluescript II SK; lane 4, E1; lane 5, K1; lane 6, K3; lane 7, K3(*Pst*I); lane 8, K3DN71; lane 9, K3DN71(*Apa*I); lane 10, K3DN105.

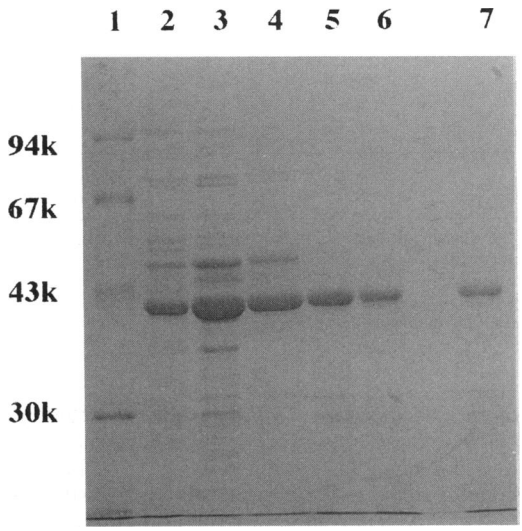

FIG. 4. SDS-PAGE of the recombinant FDH at various purification steps and of the purified native FDH. Lane 1, marker proteins (see legend to Fig. 3); lane 2, cell extract; lane 3, protamine treatment; lane 4, ammonium sulfate fraction at 45 to 70% saturation; lane 5, Toyopearl HW65C; lane 6, hydroxyapatite; lane 7, purified native FDH.

isoelectric focusing for *P. putida* FDH (2) and with that for the recombinant enzyme.

Both enzymes were active toward formaldehyde and *n*-butanol but were inactive toward ethanol. External addition of glutathione up to 2 mM did not affect the reaction rate (data not shown). Insensitivity to inhibition with 4-methylpyrazole is a unique characteristic of class III ADH (glutathione-dependent FDH) (41). Inhibition with this inhibitor was determined in 50 mM glycine-NaOH buffer (pH 8.9). Both enzymes were inhibited by this inhibitor with a K_i value of 30 μM, which is in a middle range between the values typically reported for mammalian class I and class II enzymes.

Nucleotide sequence. The nucleotide sequence of *fdhA*, from the deletion point of DN71 to the *Apa*I site, is shown in Fig. 5. There was an open reading frame consisting of 1,197 bp which began at an ATG codon. The enzyme protein is composed of 399 amino acid residues with a calculated molecular weight of 42,082. All the amino acid sequences determined by the Edman method were found in the amino acid sequence deduced from the nucleotide sequence. Consensus sequences for a ribosome binding site (Shine-Dalgarno sequence), GGAG, and a putative terminator were identified upstream from the initiation codon and at the 3′ end of the gene, respectively, but no consensus sequences for −35 and −10 regions were found. The GC content of the entire gene was 64%, which agreed with the value for *Pseudomonas* species (35). A highly AT-rich region was found around the initiation codon.

Similarity to ADH family. It is well known that zinc-containing ADHs from a wide variety of organisms are structurally related to each other (17). Recently, the glutathione-dependent FDHs from rat and human livers have been identified as class III ADHs. Comparisons were made between the amino acid sequences of *P. putida* FDH and those of ADH family enzymes from various sources, including horse liver ethanol-active ADH (horse E), the three dimensional structure of which had been solved (10), in order to clarify structural relationships. The alignment of those sequences is shown in Fig. 6. This alignment was based on comparisons of the individual sequences with the horse E isozyme structure (1, 3, 17, 18, 26), with gaps then introduced to maximize the alignment with the primary structure of *P. putida* FDH. The partial amino acid sequence of *E. coli* FDH (13) is also shown in Fig. 5. Two-thirds of the sequence from the N terminus in *P. putida* FDH showed significant homologies to all of the ADHs compared. Jörnvall et al. indicated that 22 amino acid residues were strictly conserved throughout the family (17). This number has now been reduced to 14 (1). In *P. putida* FDH, 18 of 22 residues and 12 of 14 residues, respectively, could be aligned.

TABLE 1. Purification of FDH expressed in *E. coli* DH5α harboring pFDHK3DN71

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Cell extract	2,840	19,900	7.0	100	
Protamine treatment	2,028	23,770	11.7	119	1.7
Ammonium sulfate fractionation	1,143	20,460	17.9	103	2.6
Toyopearl HW-65C	827	18,537	22.4	93	3.2
Hydroxyapatite	372	8,960	24.1	45	3.4

TABLE 2. Some properties of native and recombinant FDHs[a]

FDH	Optimum pH	pH stability[b]	Thermal stability[c]	Mol wt (10^3) determined by:		pI	K_m for formaldehyde (mM)[d]	K_i for 4-methylpyrazole (mM)[d]
				Gel filtration	SDS-PAGE			
Native	8.9	5–10	Below 60°C	150	42	5.3	0.25	0.03
Recombinant	8.9	5–11	Below 60°C	150	42	5.0	0.30	0.03

[a] For the determinations of optimum pH and kinetic parameters, FDH activity was measured by NADH formation; in other cases, diformazon formation was employed (see Materials and Methods).
[b] More than 80% of the activity remained after preincubation for 30 min at 37°C.
[c] More than 50% of the activity remained after preincubation for 30 min at pH 8.0.
[d] Values were determined at pH 8.9. Errors associated with the values were within 20%.

Most of the glycine residues (10 residues), including a Gly-Xaa-Gly-Xaa-Xaa-Gly pattern in the coenzyme binding domain, and zinc-liganding cysteine (Cys-47) and histidine (His-68) residues were conserved. Besides the active-site zinc, many medium-chain ADHs contain a structural zinc which is coordinated by four cysteine residues. These residues were also found in *P. putida* FDH (Cys-97, -100, -103, and -111). In addition to the strictly conserved residues, there were considerable similarities in regions around the active site and the coenzyme binding domain between *P. putida* FDH and the ADH family. *P. putida* FDH is significantly longer than other enzymes belonging to the medium-chain ADH family because of the long C-terminal region, which shows little alignment with any other ADHs. The overall homology of the *P. putida* FDH to ADH family enzymes ranged from 14 to 20%.

The nucleotide sequence of the *P. putida* FDH structural gene was also compared with those of various ADH structural genes. The results showed the following similarities: 45% in 896 bases, 42% in 1,025 bases, 46% in 1,046 bases, 46% in 997 bases, 43% in 738 bases, 44% in 1,055 bases, and 45% in 994

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                                                                  TGACCCTGGCCAAAGCCGTCGGCTACCAC  -268
ATCGAACGCCGGGTGTTCCTCAACGCCAACCGCACGGTGGTGCTCAACGCTTGAGGCTTTTCCCCTCTCCCTTCCGGGAGAGGGTCGGGG  -178
GAGAGGGTGCGCCTTCTTCCGTCAGATTCAATGGTGCGCACGGCGCACCCTGCTTTCACGGAGTCCGCCTCTGCCAGGTTTCACCGGTGG   -88
GCTCCGGCAACAGACGCAAGCTCCAGTGCAAGGCCGCCTGGCCGTCGGTCGCGCCTTTGTATTCACAACAACAACGGAGAATTACGCATG     3
                                                                          S.D.        M
TCTGGTAATCGTGGTGTCGTTTATCTCGGTTCGGGCAAGGTCGAAGTCCAGAAGATCGACTACCCCAAAATGCAGGACCCTCGCGGCAAG    93
 1 S G N R G V V Y L G S G K V E V Q K I D Y P K M Q D P R G K
AAGATCGAGCACGGGGTCATCCTGAAAGTCGTCTCCACCAACATCTGCGGCTCGGACCAGCACATGGTGCGCGGTCGTACCACCGCCCAG   183
31 K I E H G V I L K V V S T N I C G S D Q H M V R G R T T A Q
GTAGGCCTCGTGCTTGGCCACGAGATCACCGGTGAGGTGATCGAGAAGGGCCGTGACGTAGAGAACCTGCAGATCGGCGACCTGGTTTCC   273
61 V G L V L G H E I T G E V I E K G R D V E N L Q I G D L V S
GTACCCTTCAACGTGGCCTGCGGCCGCTGCCGTTCCTGCAAGGAAATGCACACCGGCGTTTGCCTGACCGTCAATCCGGCCCGCGCTGGC   363
91 V P F N V A C G R C R S C K E M H T G V C L T V N P A R A G
GGCGCCTACGGCTATGTCGACATGGGCGACTGGACCGGTGGCCAGGCCGAGTACTTGCTGGTGCCCTACGCCGACTTCAACCTGCTCAAG   453
121 G A Y G Y V D M G D W T G G Q A E Y L L V P Y A D F N L L K
CTGCCGGATCGCGACAAGGCCATGGAGAAGATCCGTGACCTGACCTGCCTTTCCGACATCCTGCCCACCGGCTACCACGGCGCCGTAACT   543
151 L P D R D K A M E K I R D L T C L S D I L P T G Y H G A V T
GCCGGCGTTGGCCCGGGCAGCACCGTGTACGTGGCCGGTGCCGGCCCGGTCGGCCTGGCCGCTGCCGCCTCCGCTCGCCTGCTGGGCGCC   633
181 A G V G P G S T V Y V A G A G P V G L A A A A S A R L L G A
GCCGTGGTTATCGTCGGCGACCTCAACCCCGCCCGACTGGCCCACGCCAAGGCGCAGGGCTTCGAGATCGCCGACCTGTCGCTGGACACC   723
211 A V V I V G D L N P A R L A H A K A Q G F E I A D L S L D T
CCGCTGCACGAGCAGATCGCTGCGCTGCTGGGAGAGCCGGAAGTGGATTGCGCGGTGGATGCGGTGGGCTTCGAAGCTCGCGGCCACGGC   813
241 P L H E Q I A A L L G E P E V D C A V D A V G F E A R G H G
CACGAAGGTGCCAAGCACGAAGCCCCGGCCACTGTGCTGAACTCGCTCATGCAAGTCACCCGCGTGGCCGGCAAGATCGGTATCCCCGGC   903
271 H E G A K H E A P A T V L N S L M Q V T R V A G K I G I P G
CTTTACGTCACCGAAGATCCGGGTGCCGTGGACGCCGCGGCGAAGATCGGCAGCCTGAGTATTCGCTTCGGCCTCGGCTGGGCGAAATCC   993
301 L Y V T E D P G A V D A A A K I G S L S I R F G L G W A K S
CACAGCTTCCACACTGGCCAGACCCCGGTGATGAAGTACAACCGCGCACTCATGCAAGCGATCATGTGGGACCGCATCAATATCGCCGAA  1083
331 H S F H T G Q T P V M K Y N R A L M Q A I M W D R I N I A E
GTGGTGGGCGTGCAGGTCATCAGCCTGGACGACGCACCGCGTGGCTATGGCGAGTTCGATGCCGGCGTGCCGAAGAAGTTCGTCATCGAC  1173
361 V V G V Q V I S L D D A P R G Y G E F D A G V P K K F V I D
CCGCACAAGACCTTCAGCGCGGCCTGATCGACTGCAATACCCCCGACGCCGTGATGTGACAGCCGCGGCGTCTGTCCCTCAAGGGGCCCT  1263
391 P H K T F S A A *
TCGGGGCCCTTTTTTTGTATCCGGCGAAAAGTAGTAAACCTGCAAGGCATCATTCTTCTTCCAGTTCGACAAAGCGCTCGAGACTCACCA  1353
TATCAGCTTGATCCGCC
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FIG. 5. Nucleotide sequence of the FDH gene from *P. putida* and deduced amino acid sequence of the enzyme. The nucleotide sequence is numbered from the initiation codon. Dashed underlining shows the Shine-Dalgarno sequence (S. D.) and the putative transcription terminator. Italics indicate the 485-bp PCR product. Numbering of the amino acid sequence starts from the amino terminus of the mature enzyme. Amino acid sequences determined by the Edman method are indicated by underlining.

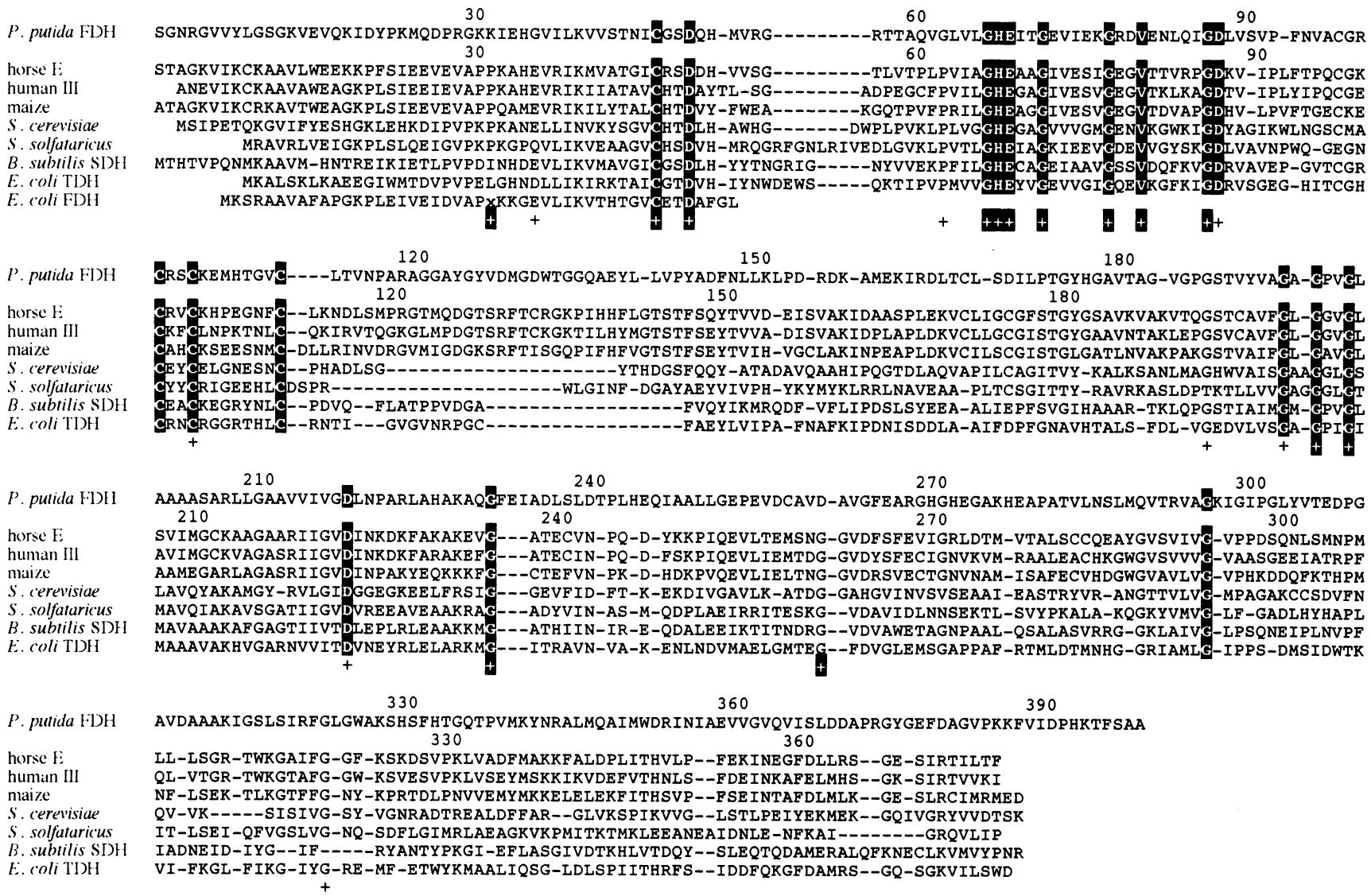

FIG. 6. Alignment of the amino acid sequence of *P. putida* FDH with those of members of the ADH family. Numbers above the amino acid sequences refer to *P. putida* FDH and horse liver E-type ADH (class I), as indicated. Strictly conserved residues within the family (17) are indicated by + at the bottom of the sequences; + shown against a black background indicates the 14 residues which are still conserved in some bacterial ADHs (1). Gaps introduced to maximize alignment are shown by −. Residues that occur in all enzymes are given against a black background. Sequence data for horse E, maize ADH2, and *S. cerevisiae* ADH1 are from reference 17, and those for human class III ADH are from reference 18. Bacterial ADH structures are from references 1, 3, and 26. For *E. coli* glutathione-dependent FDH, only 47 residues from the N terminus are shown (13). From this alignment, homology percentages were calculated by dividing the number of identical amino acid residues at the corresponding positions by the total number of amino acid residues of *P. putida* FDH (388 residues), without considering gaps and insertions in the calculations. SDH, sorbitol dehydrogenase; TDH, threonine dehydrogenase.

bases with horse E isozyme (29), human class III ADH (12), maize ADH2 (6), *S. cerevisiae* ADH1 (4), *Sulfolobus solfataricus* ADH (1), *Bacillus subtilis* sorbitol dehydrogenase (26), and *E. coli* threonine dehydrogenase (3), respectively. These values were comparable to or slightly lower than those obtained from comparisons of the ADH structural genes from microbes other than *P. putida*.

DISCUSSION

We have cloned the FDH gene (*fdhA*) from *P. putida* in *E. coli* DH5α cells and determined its nucleotide sequence. This is the first report about the sequence of the glutathione-independent FDH. *P. putida* FDH was found to be encoded by an open reading frame of 1,197 bp, giving a protein of 399 amino acid residues. The partial amino acid sequences determined by protein sequencing agreed well with those deduced from the nucleotide sequence, except that the mature enzyme lacks methionine encoded by the initiation codon.

The transformant that carried a 2.1-kb fragment inserted into pBluescript showed much greater FDH activity than *P. putida*. The plasmid pFDHK3DN71 under the control of the *lac* promoter on the vector could express the enzyme about 50-fold more than *P. putida*. Amounts of FDH protein could be nearly 30% of the total protein found in cell extracts of *E. coli* DH5α harboring pFDHK3DN71, as judged by SDS-PAGE. The expressed enzyme had a molecular weight and physicochemical and catalytic properties that were almost the same as those of the native enzyme.

Zinc-containing ADHs are structurally related to each other, forming a large family of enzymes, the so-called medium-chain ADH family. Recently, glutathione-dependent FDHs from human and rat livers have been identified as class III ADHs, which are less variable than the traditional liver enzyme of class I (19) and are considered to be predecessors of other classes (5). *P. putida* FDH is also a zinc-containing metalloenzyme (27) and showed significant homologies to ADHs of various origins, including several bacterial ADHs. There were no significant differences between the homologies with mammalian ADHs of class I and class III (18 versus 17%). Although the overall homology to each ADH compared was relatively low (less than 20%), alignment revealed distant but clear structural relationships between them.

Each class of the mammalian ADH family has been defined on the basis of its functional and physicochemical properties (36). Class I ADH is cationic and highly sensitive to inhibition by 4-methylpyrazole. Class II ADH is also cationic but is less sensitive to the inhibitor. Class III ADH is the only anionic protein and is insensitive to 4-methylpyrazole. Class I and II ADHs show activity toward ethanol, while class III ADH shows glutathione-dependent FDH activity but is almost inactive toward ethanol. Glutathione- or factor-dependent FDHs from *E. coli* (13), *Amycolatopsis methanolica* (40), and *Rhodococcus erythropolis* (7) also behave as ADH class III enzymes, oxidizing and reducing higher aliphatic alcohols and aldehydes, respectively. Functionally, *P. putida* FDH is similar to class III ADH (glutathione-dependent FDH); it can catalyze the NAD^+-dependent oxidation of long-chain alcohols such as *n*-pentanol but is inactive toward ethanol at high pH values. These bacterial enzymes are also acidic proteins with isoelectric points of between 4 and 7. Strong inhibition by 4-methylpyrazole, however, was observed only for *P. putida* FDH (K_i = 30 μM). The sensitivity to 4-methylpyrazole has been suggested to be related to conditions at position 51 in horse liver E enzyme (9). All the class I enzymes, which have His-51, are strongly inhibited, while class III enzymes, which have Tyr-51, are almost insensitive. Little is known about other bacterial FDHs with respect to the primary structures, except for the enzyme presented in this paper. So far, the enzyme from *P. putida* C-83, which has His-51 as in class I ADH, is the only FDH that is strongly inhibited by this inhibitor. Thus, *P. putida* FDH showed mixed properties in relation to mammalian ADHs of class I and class III.

There are several kinds of enzymes capable of oxidizing formaldehyde. These include aldehyde dehydrogenases (16, 39), formaldehyde dismutase (21), glutathione- or factor-dependent FDHs (class III ADHs) (38, 40), and glutathione-independent FDH from *P. putida*. Aldehyde dehydrogenases are nonspecific enzymes; class III ADHs require a helper substrate (glutathione or factor), producing a formyl ester. In this respect, it can be said that the enzyme from *P. putida* is a genuine FDH.

P. putida FDH is markedly inhibited by PCMB (28). There are seven cysteine residues in the sequence, and five of them seem identical to those of horse E, whose three-dimensional structure has been crystallographically analyzed. Cys-174 of the horse ADH has been shown to coordinate to the active-site zinc. In the case of *P. putida* FDH, however, aspartic acid (Asp-169) was aligned at this position (Fig. 6). On the basis of computer modeling, it was suggested that the sheep liver sorbitol dehydrogenase had a glutamic acid (Glu-174) as the third active-site zinc ligand (8). This idea has been supported by a recent site-directed mutagenesis experiment (20). In *E. coli* threonine dehydrogenase (3) and *Thermoanaerobium brockii* ADH (1), an aspartate residue was found in the place of Glu-174 of sheep liver sorbitol dehydrogenase. Nothing is yet known about the zinc coordination of *P. putida* FDH, but either Cys-166 or Asp-169 might be the third ligand of the active-site zinc. The seventh cysteine residue in *P. putida* FDH is Cys-257, which seems to be unique to *P. putida* FDH.

Ogushi et al. indicated that one of the PCMB-reactive sulfhydryl groups is involved in the binding of formaldehyde to the enzyme (28). This sulfhydryl group probably behaves as an internal glutathione to form a thiohemiacetal as an intermediate in a way proposed for aldehyde dehydrogenase (16). We could not identify the reactive cysteine residue homologous to the active-site cysteine of aldehyde dehydrogenase by sequence comparison. The reaction mechanism of *P. putida* FDH could be different from that of aldehyde dehydrogenase. The functions of five of seven cysteine residues in *P. putida* FDH, as mentioned above, are likely to be the same as in horse liver ADH. The roles of Cys-166, Cys-257, and Asp-169 are now being investigated by site-directed mutagenesis to determine the third ligand of the active-site zinc and the reactive cysteine residue.

Mammalian ADHs are usually dimeric proteins. The glutathione-dependent FDH from *E. coli* was reported to be a homodimer (13), while *P. putida* FDH exists as a tetrameric form like other bacterial ADHs (1, 39). ADHs from yeasts and sheep liver sorbitol dehydrogenase are also known to be tetrameric and lack the region corresponding to the residues 119 to 139 in horse liver ADH (8, 15). *P. putida* FDH has this region, but its amino acid sequence is barely homologous to those of dimeric ADHs. The region(s) involved in subunit interaction in *P. putida* FDH remains to be determined.

In conclusion, glutathione-independent FDH from *P. putida* belongs to the zinc-containing medium-chain ADH family, although there are extensive variations in the amino acid sequence. The cloned gene and the high-level expression system will be useful to clarify the zinc coordination in *P. putida* FDH and the mechanism of glutathione-independent dehydrogenation of formaldehyde by the enzyme.

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