Cloning, Nucleotide Sequencing, and Expression in *Escherichia coli* of the Gene for Formate Dehydrogenase of *Paracoccus* sp. 12-A, a Formate-assimilating Bacterium

Takeshi SHINODA, Tamao SATOH, Shigeru MINEKI, Mitsugi IIDA, and Hayao TAGUCHI

Department of Applied Biological Science, Faculty of Science and Technology, Science University of Tokyo, Noda, Chiba 278-8510, Japan

Received July 27, 2001; Accepted September 24, 2001

The gene for the NAD-dependent formate dehydrogenase (FDH) of *Paracoccus* sp. 12-A, a formate-assimilating bacterium, was cloned through screening of the genomic library with activity staining. The FDH gene included an open reading frame of 1,200 base pairs, and encoded a protein of 43,757 Da, which had high amino acid sequence identity with known FDHs, in particular, with bacterial enzymes such as those of *Moraxella* sp. (86.5%) and *Pseudomonas* sp. 101 (83.5%). The gene was highly expressed in *Escherichia coli* cells using an expression plasmid with the pUC ori and tac promoter. The recombinant enzyme was somewhat inactive in the stage of the cell-free extract, but its activity markedly increased with purification, in particular, with the step of heat-treatment at 50°C. The purified enzyme showed essentially the same properties as the enzyme from the original *Paracoccus* cells.

Key words: formate dehydrogenase; *Paracoccus*; activity staining; formate-assimilating bacterium; NAD-dependent dehydrogenase

NAD-dependent formate dehydrogenases (FDHs) (EC 1.2.1.2), which catalyze the conversion of formate to carbon dioxide with the concomitant reduction of NAD⁺ into NADH, have been found in various organisms such as plants, methylotrophic yeasts, and bacteria. In methylotrophic yeasts and bacteria, FDHs play a key role in the catabolism of C1 compounds such as methanol by catalyzing the final step. On the other hand, FDH is one of the most promising enzymes for NADH regeneration in multi-enzyme systems such as bioreactors involving NADH as a coenzyme, since the equilibrium of the reaction with FDH lies in the direction of NADH generation from NAD⁺, unlike the other usual NAD-dependent dehydrogenases. Bacterial FDHs are usually advantageous for such applications because of their higher stability than that of the enzymes from other organisms. Among bacterial FDHs, in particular, the three-dimensional structure and structure-function relationship of *Pseudomonas* sp. 101 enzyme has been well studied.

*Paracoccus* sp. 12-A, which was isolated from sewage as a formate-assimilating and FDH-producing bacterium, can use formate as a sole carbon source for growth, but hardly uses methanol, unlike the usual methyrotrophic bacteria with FDHs. The *Paracoccus* FDH, which is significantly induced within cells when they are grown in a formate-containing medium, has been purified from *Paracoccus* cells, and the purified enzyme had high specific activity and stability, so it may also be promising for application. In the process of further study, however, we realized that the enzyme in the cell-free extract of *Paracoccus* cells is much more labile than the purified enzyme sample, so it can hardly be purified reproducibly. This suggests that *Paracoccus* cells may contain some factor(s) that promote inactivation of the enzyme. In this paper, we describe the molecular cloning, nucleotide sequence, and high expression of the *Paracoccus* FDH gene in *Escherichia coli* cells, by which a large amount of the active recombinant FDH could readily be purified.

Materials and Methods

Construction of a *Paracoccus* sp. 12-A genome library. Chromosomal DNA was isolated from the *Paracoccus* sp. 12-A cells, which were cultivated at 30°C as described in our previous paper, essentially according to Saito and Miura. After partial digestion with *Sau*3AI and electrophoresis on 0.8% agarose, the *Sau*3AI-fragments (about 2.0 to 4.0-kb) of DNA were collected from the agarose gel, ligated with a ZAP Express Predigested (BamHI/CIAP-treated) Vector (Stratagene), and then packaged into phage particles with Gigapack III Gold packaging.
extracts (Stratagene). The phage library obtained was converted into a colony library containing recombinant pBK-CMV phagemids by in vivo excitation with a ZAP Express Predigested Vector kit (Stratagene).

Activity staining. The E. coli cells with recombinant pBK-CMV phagemids were plated onto LB agar plates containing kanamycin of 50 mg/ml (200 to 300 colonies/plate). When colonies had grown to 1 to 2 mm in diameter, they were transferred to Hybond-N+ nylon membranes (Amersham Pharmacia Biotech). After incubation on fresh plates at 37°C overnight and then at 4°C for 4 h, the nylon membranes were soaked in a thin layer of 50 mM sodium phosphate buffer containing 1 mM EDTA and 20 mg/ml hen-egg lysozyme, and then incubated at 37°C for 3 h. After drying on filter papers, the membranes were placed onto 2 mm in diameter, they were transferred to Hybond-N+ nylon membranes (Amersham Pharmacia Biotech). After incubation on fresh plates at 37°C overnight and then at 4°C for 4 h, the nylon membranes were soaked in a thin layer of 50 mM sodium phosphate buffer containing 1 mM EDTA, 0.05 mg/ml phenazinemethosulfate, and 50 mM sodium phosphate buffer (pH 7.0), and then incubated at 37°C.

Nucleotide sequence analysis and site-directed mutagenesis. DNA sequencing was done by the dideoxy chain terminator procedure with an Applied Biosystems 373A DNA sequencer model 4000L (LI-COR). Oligonucleotide 5'-CTATGACATGTTCGATATCTGCTTCTTTCGGTTC-3' was obtained from Takara Shuzo (Kyoto), and used to introduce an EcoRI site into the 5'-flanking sequence of the obtained FDH gene by site-directed mutagenesis. The 1.2-kb DNA insert from the 2.0-kb insert was then subcloned into the EcoRI-NruI segment was inserted between the EcoRI and SmaI sites of pKM (Fig. 1).

Purification of the recombinant FDH. The E. coli MV1184 cells carrying the expression plasmid were aerobically grown at 37°C in the presence of 150 μg/ml ampicillin in a liquid culture of 2×YT medium (1.6% tryptone, 1.0% yeast extract, and 0.5% NaCl) containing 0.5% and 0.2% glucose, which significantly improved the cell growth (data not shown), for the pre-culture and large-scale culture (one liter) of the cells, respectively. When the optical density at 600 nm of the culture reached 0.5 to 1.0, the culture (one liter) of the cells, respectively. When the optical density at 600 nm of the culture reached 0.5 to 1.0, the culture (one liter) of the cells, respectively. When the optical density at 600 nm of the culture reached 0.5 to 1.0, the culture was harvested by centrifugation and washed with 10 mM sodium formate and 0.2 M for formate and 0.2 M for NAD+ was obtained in the absence of inhibition codon could be positioned at an appropriate distance from the SD sequence when the EcoRI-NruI segment was inserted between the EcoRI and SmaI sites of pKM (Fig. 1).

Enzyme assay and protein measurement. The standard enzyme assays were done at 30°C in 100 mM sodium phosphate buffer (pH 7.0) containing 1 mM NAD+ and 20 mM sodium formate. One unit was defined as the catalytic rate of conversion of one μmol of substrate per one minute. Protein concentrations were measured with Bio-Rad Protein Assay protein reagent by Bradford's method, using bovine serum albumin as a standard protein. The purity of the enzyme preparations was examined by SDS-PAGE using Laemmli’s method. Kinetic parameters of the purified recombinant Paracoccus FDH were obtained from plots of [S]/v versus [S]. The Km for formate and Vmax were calculated from the standard assay mixture containing various concentrations of formate, and the Km for NAD+ was obtained in the presence of 20 mM formate and various concentrations of NAD+.

Construction of an expression plasmid for the Paracoccus sp. 12-A FDH gene. Figure 1 summarizes the construction of an expression plasmid for the ORF for the cloned FDH gene, using one of the cloned DNA inserts (2.0-kb) that contain the FDH gene and an expression vector, pKM. pKM was derived from an inducible expression vector, pKUM, which was constructed from pKK223-3 (lac promoter, Shine-Dalgarno sequence, multicloning site, rrn-BT1BT2 and NH2-terminal region of β-lactamase), pUC18 (ori, COOH-terminal region of β-lactamase), and pMJR1560 (lacIq), only by replacing the multicloning site of pKK223-3 with that of pUC18. A unique EcoRI site was introduced into the 5'-flanking sequence of the gene, so that the initiation codon could be positioned at an appropriate distance from the SD sequence when the EcoRI-NruI segment was inserted between the EcoRI and SmaI sites of pKM (Fig. 1).

Fig. 1. Construction of an Expression Plasmid for the Cloned FDH Gene.

Boxes indicate the cloned 2.0-kb DNA insert, in which the ORF for the FDH is shadowed. Thick arrows within the shadowed boxes indicate the direction of the transcription for the ORF. The cloned 2.0-kb DNA insert in phagemid pBK-CMV (see Fig. 2) was subcloned into pUC18 by using unique PstI and HindIII restriction sites on both vectors, and then a unique EcoRI site was introduced into the 5'-flanking sequence of the ORF by site-directed mutagenesis. The 1.2-kb EcoRI-NruI fragment from the 2.0-kb insert was then subcloned into the EcoRI and SmaI sites of the pKM plasmid vector.
0.6, IPTG was added to the culture medium (final concentration = 0.5 mM). After further cultivation at 30°C for 10 h, cells were harvested by centrifugation, washed, and resuspended in 10 mM sodium phosphate buffer (pH 7.0), and then disrupted by sonication on ice. The crude cell-free extract was heated at 50°C for 30 min and then centrifuged. The clear supernatant obtained was put on a column of DEAE-Sephacel gel (Amersham Pharmacia Biotech), and the fraction containing FDH activity was eluted with a stepwise increase in the sodium phosphate buffer concentration from 50 mM to 100 mM. After the addition of ammonium sulfate to a final 20% saturation and filtration, the enzyme sample fraction was put onto a column of Butyl Toyopearl 650M (Tosoh, Tokyo), which was equilibrated with 100 mM sodium phosphate buffer (pH 7.0) containing 20% saturated ammonium sulfate, and then eluted with a reduction in the ammonium sulfate concentration to 0% saturation. Both column chromatographies were done at room temperature.

Results and Discussion

Cloning and sequencing of the gene for Paracoccus sp. 12-A FDH

On activity staining, two colonies were a clear blue in color among the about 3,000 colonies comprising the constructed Paracoccus sp. 12-A genomic library. These two clones contained phagemids with inserts of 2,000 and 4,500 bp, respectively. Restriction mapping and sequence analysis revealed that the two DNA clones share a common DNA region of 1,345 bp that has Sau3AI restriction sites at the two termini (DDBJ Accession number AB071373), as shown in Fig. 2, but differ from each other in the other regions of the inserted DNA (data not shown). Southern hybridization with a probe of the 0.9-kb BssHII fragment within the region (Fig. 2) indicated that there is only a single copy of this DNA segment in the Paracoccus sp. 12-A chromosome (data not shown), suggesting that the common 1,345-bp region in the two clones originated from the same DNA area on the chromosome, through ligation with different Sau3AI fragments during the construction of the genomic library.

The nucleotide sequence of the common 1,345-bp segment is shown in Fig. 2, together with the following sequence up to NruI site of the isolated 2.0 kb clone, which was used to construct an expression plasmid (Fig. 1). The sequence contained only one possible open reading frame (ORF) for the FDH protein. The ORF comprised 1.2 kb with an ATG initiation codon and a TGA termination codon, and encoded a protein of 43,757 Da (400 a.a.), which is similar to the reported subunit molecular mass (4.9 kDa) of the Paracoccus FDH on SDS-PAGE.15) While there was a possible Shine-Dalgano (SD) sequence in the 5'-flanking region of the FDH gene, no possible promoter site that acts in E. coli was found up to 230 bp upstream of the FDH gene (Fig. 1). On the other hand, a putative Paracoccus promoter region (−35 and −10 regions) was boxed.

Purification of the recombinant Paracoccus FDH

The recombinant FDH was sufficiently stable in the cell-free extract of E. coli cells to be readily purified from the extract only through the three purification steps, i.e. heat-treatment, and anion-exchanging and
hydrophobic chromatographies at room temperature (Table 1 and Fig. 3). The purified enzyme sample gave a single protein band on SDS-PAGE (Fig. 3), and had about the 12-fold increase in specific activity over the crude extract (Table 1). In the case of the cell-free extract of the original Paracoccus sp. 12-A cells, in contrast, the FDH activity was rapidly lost even when the purification was done at 4°C, and therefore the enzyme could hardly be purified reproducibly, although the inactivation rate somewhat varied depending on the cell cultivation and crude extract preparation (data not shown). These results suggest that Paracoccus sp. 12-A cells may contain some factor(s) that stimulates the inactivation of FDH, but that E. coli cells may not.

SDS-PAGE of the crude extract gave an apparently stronger protein band of FDH, i.e. more than 8% of total protein (Fig. 3), which can be simply estimated from the 12-fold increase in the specific activity during the purification (Table 1). On the other hand, the total activity of FDH markedly increased by 1.5-fold in the heat-treatment step (Table 1). In addition, for the cell-free extract sample, the enzyme reaction showed a marked lag period in the progress curve of the time-dependent increase in absorbance at 340 nm of NADH produced, before developing an apparently linear progress curve (data not shown), from which the enzyme activity was estimated in the case of the crude extract. Such a lag period clearly disappeared after treatment of the sample at 50°C for 30 min. These results suggest that the amount of recombinant FDH protein produced is at least more than 12% (1.5 x 8%) of the total soluble proteins in E. coli cells, but is somewhat inactive or inhibited by unknown factor(s) within E. coli cells in the crude extract. The assay at high temperature conditions may reduce the inhibitory effect of such a factor or induce some conformational change in the FDH protein.

**Characteristics of the recombinant FDH**

The finally purified recombinant enzyme had similar specific activity (7.8 units/mg) (Table 1) to that reported for the enzyme from the original Paracoccus cell (11.6 units/mg), suggesting that it is as active as the original enzyme. Table 2 shows the kinetic parameters for the purified recombinant enzyme. The $K_M$ values for formate and NAD$^+$ of the recombinant enzyme were virtually the same as the reported values for the original Paracoccus enzyme. The recombinant enzyme showed no loss, and no further increase, in its activity on heat-treatment for 1 h up to 50°C, but only about 25% of the activity remained after treatment at 55°C for 1 h. The enzyme had the maximal catalytic reaction at 55°C, and the optimal pH for the catalysis was in the range of 5.5 to 7.0. These properties of the recombinant FDH are also essentially consistent with those of the enzyme from the original Paracoccus cells.

**Amino acid sequence comparison of the Paracoccus sp. 12-A FDH with other FDHs**

Among known FDHs, the Paracoccus sp. 12-A FDH exhibited higher sequence identity to the bacterial FDHs (more than 80%) from Moraxella sp. (Galkin, A. G., unpublished result, EMBL Y13245-1), Pseudomonas sp. 101, and Mycobacterium vaccae N10, than other FDHs (40 to 50%) from eucaryotes, such as plants and yeasts. Figure 4 and Table 3 show amino sequence alignment and the mutual sequence identity among the bacterial FDHs, respectively. The sequence of the Paracoccus enzyme

---

### Table 1. Purification of Recombinant Paracoccus sp. 12-A FDH

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>454</td>
<td>707</td>
<td>0.64</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Heat treatment</td>
<td>685</td>
<td>559</td>
<td>1.22</td>
<td>151</td>
<td>1.9</td>
</tr>
<tr>
<td>DEAE Sephacel</td>
<td>649</td>
<td>147</td>
<td>4.44</td>
<td>143</td>
<td>6.9</td>
</tr>
<tr>
<td>Butyl Toyopearl</td>
<td>343</td>
<td>44</td>
<td>7.82</td>
<td>76</td>
<td>12.2</td>
</tr>
</tbody>
</table>

### Table 2. Kinetic Parameters for Paracoccus sp 12-A FDH

<table>
<thead>
<tr>
<th>FDHs</th>
<th>$K_M$ (formate) (mM)</th>
<th>$K_M$ (NAD$^+$) (mM)</th>
<th>$V_{max}$ (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinant enzyme</td>
<td>5.6</td>
<td>54</td>
<td>8.3</td>
</tr>
<tr>
<td>Paracoccus enzyme$^{15}$</td>
<td>5.0</td>
<td>36</td>
<td>—</td>
</tr>
</tbody>
</table>

---

**Fig. 3.** SDS-Polyacrylamide Gel Electrophoresis Showing the Purity of the Recombinant FDH at Each Purification Step.

Lane 1, standard marker proteins (Amersham Pharmacia Biotech); lane 2, crude extract of E. coli MV1184 cells transformed with pKM containing the Paracoccus sp. 12-A FDH gene; lane 3, heat-treatment pool; lane 4, DEAE-Sephacel pool; lane 5, Butyl Toyopearl pool.
Fig. 4. Comparison of the Amino Acid Sequences of Bacterial FDHs.  
1. Paracoccus sp. 12-A FDH; 2. Moraxella sp. FDH (EMBL Y13245-1); 3. Pseudomonas sp. 102 FDH; 4. Mycobacterium vaccae N10 FDH. The numbering of the amino acids is according to Pseudomonas sp. 101 FDH. The same amino acids to those in Paracoccus sp. 12-A FDH are marked in black.

Table 3. Amino Acid Sequence Identity among Bacterial FDHs (%)

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Paracoccus sp. 12-A</td>
<td>100.0</td>
<td>86.5</td>
<td>83.5</td>
<td>83.3</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>Catalytic domain</td>
<td>Coenzyme-binding domain</td>
<td></td>
</tr>
<tr>
<td>2. Moraxella sp.</td>
<td>100.0</td>
<td>86.3</td>
<td>83.0</td>
<td>89.3</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>Catalytic domain</td>
<td>Coenzyme-binding domain</td>
<td></td>
</tr>
<tr>
<td>3. Pseudomonas sp. 101</td>
<td>100.0</td>
<td>99.5</td>
<td>99.1</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>Catalytic domain</td>
<td>Coenzyme-binding domain</td>
<td></td>
</tr>
<tr>
<td>4. Mycobacterium vaccae N10</td>
<td>100.0</td>
<td>86.5</td>
<td>86.3</td>
<td>83.5</td>
</tr>
</tbody>
</table>

The amino acids of the catalytic domain (positions 1–146 and 334–400) and coenzyme-binding domain (positions 147–333) are according to Pseudomonas sp. 101 FDH.  

Formate Dehydrogenase Gene of Paracoccus sp. 12-A could be aligned with those of the known bacterial enzymes without any deletion or insertion (Fig. 4). Many amino acids that are involved in the enzyme function have been identified or suggested for Pseudomonas sp. 101 FDH, for example, Asn146 and Arg284 are proposed to be essential for substrate binding and the subsequent catalytic action of the enzyme. The Paracoccus enzyme apparently has no lack or replacement of such amino acids important for the enzyme function. In addition, for the Pseudomonas enzyme, it has been reported that the stability of the enzyme is significantly increased by replacements for low conserved serine residues at positions 131, 160, 184, and 228 with Ala, a more hydrophobic residue. None of these Ser residues are conserved in the Paracoccus enzyme, as in the case of many other FDHs, but are replaced with hydrophilic residues such as Asn, Thr, and Glu, except for the case of Ser131 (Fig. 4). It is also known that the replacement of Ser168, which is also poorly conserved in known FDHs, with Ala rather reduces the stability of the Pseudomonas enzyme, in which the Ser168 OH-group forms a hydrogen bond with Asn164. Together with Asn164, Ser168 is conserved in the known bacterial FDHs including the Paracoccus enzyme.

While the Mycobacterium and Pseudomonas enzymes are closely related to each other (only two amino acid residues are different), the Paracoccus enzyme is markedly different from all three known bacterial enzymes in the sequence (Table 3). Among these bacterial FDHs, nevertheless, the Paracoccus and Pseudomonas (or Mycobacterium) enzymes have slightly higher sequence identity with the Moraxella enzyme (86.5% and 86.3%, respectively) than their mutual identity (83.5%) (Table 3). It was also notable that the Paracoccus and Pseudomonas enzymes...
showed higher similarity with the *Moraxella* enzyme in the distinct protein locus. Between the *Paracoccus* and *Moraxella* enzymes, for example, the similarity in the catalytic domains (89.7%) is markedly higher than that in the coenzyme-binding domains (82.5%), while the coenzyme-binding domains are more similar (89.3%) than the catalytic domains (83.9%) between the *Moraxella* and *Pseudomonas* enzymes. Thus, the *Paracoccus* and *Pseudomonas* (or *Mycobacterium*) enzymes appear to be farther distant among the three groups of bacterial FDHs.

**Acknowledgment**

This work was supported by a Grant-in-Aid for Scientific Research to H. T. from the Ministry of Education, Science, Sports, and Culture of Japan.

**References**