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SHORT CONTRIBUTION

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Cloning of formate dehydrogenase gene from a methanol-utilizing bacterium *Mycobacterium vaccae* N10

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Abstract The gene of NAD^+-dependent formate dehydrogenase (FDH) from *Mycobacterium vaccae* N10 was cloned into *Escherichia coli* by hybridization with digoxigenin-labeled DNA probes, which were prepared by amplification of the chromosomal DNA from the bacterium by the polymerase chain reaction with degenerate primers. The primers were designed on the basis of the most conserved parts of known sequences of FDH from different organisms. An open-reading frame of 1200 bp exhibited extremely high sequence similarity to the FDH gene of *Pseudomonas* sp. 101. The deduced amino acid sequence of FDH from *Mycobacterium vaccae* N10 (McFDH) was identical to that of *Pseudomonas* sp. 101 (PsFDH) except for two amino acid residues: isoleucine-35 (threonine in PsFDH) and glutamate-61 (lysine in PsFDH). The physicochemical properties of both enzymes appeared to be closely similar to each other, but the thermostability of McFDH was a little lower than that of PsFDH. To examine the role of the two amino acid residues in the thermostability of the enzymes, glutamate-61 of McFDH was replaced by glutaminyl, prolyl and lysyl residues by site-directed mutagenesis. All the mutant enzymes showed higher thermostability than the wild-type McFDH. The negative charge of glutamate-61 contributes to the stability of the wild-type enzyme being lower than that of PsFDH.

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Introduction

NAD^+-dependent formate dehydrogenase (FDH, EC 1.2.1.2), catalyzing the oxidation of formate to CO_2, is found in various organisms, in particular methanol-utilizing yeasts and bacteria (Izumi et al. 1989; Schutte et al. 1976; Kato et al. 1974; Hou et al. 1982; Tishkov et al. 1989; Egorov et al. 1979; Asano et al. 1988). Bacterial FDH are stable and useful as catalysts for NADH regeneration in multi-enzyme systems (Ohshima et al. 1985, 1989). The FDH gene of the methylotrophic bacterium *Pseudomonas* sp. 101 was cloned and sequenced (Tishkov et al. 1991). The tertiary structure of the enzyme (PsFDH) was analyzed at high resolution (Lamzin et al. 1994). Attempts have been made to increase the thermostability of PsFDH by site-directed mutagenesis. Replacement of cysteine-255 by a serine residue gave a mutant enzyme that was stable to air oxidation, but the mutant enzyme was less thermostable than the wild-type enzyme (Tishkov et al. 1993). Mutant PsFDH with a new coenzyme specificity acting on $NADP^+$ as the preferred coenzyme were also prepared (for review, see Popov and Lamzin 1994). However, none of them was more thermostable than the wild-type enzyme.

We have found another methanol-utilizing bacterium *Mycobacterium vaccae* N10 producing an FDH (McFDH). The enzyme resembles PsFDH in various physicochemical and catalytic properties except for thermostability: McFDH is less thermostable than PsFDH. The deduced amino acid sequence of McFDH differs from that of PsFDH only at two positions: isoleucine-36 (threonine in PsFDH) and glutamate-61 (lysine in PsFDH). We have examined the role of these amino acid residues on the thermostability of the enzymes by means of site-directed mutagenesis, and found that the negative charge of glutamate-61 contributes to the lower thermostability of McFDH. We here describe cloning and DNA sequencing of the McFDH gene, and also the results of the site-directed mutagenesis.

Materials and methods

Enzymes, chemicals, bacteria, and plasmids

Commercial enzymes were purchased from Toyobo or Takara, Japan and used according to the manufacturer's instructions. NAD^+ was from Serva, and all other chemicals were from Nacalai Tesque or Wako Pure Chemicals, Japan. Hybond-N transfer membranes used in hybridization experiments were from Amersham. Oligonucleotides were synthesized with an Applied Biosystems 380A DNA synthesizer or purchased from Biologica, Nagoya, Japan.

Mycobacterium vaccae N10 was obtained from the culture collection of the Biology Department of Moscow State University, Russia. The bacterial cells were cultured in a methanol-containing medium and the cultivation conditions were essentially the same as described previously (Egorov et al. 1979). *E. coli* TG1 [F′(*traD36*, *proAB*, *lacIQ*, *lac2* M15) Δ(*lac-pro*), *thi*$^-$, *hsdR*, *ara*$^-$] and a vector plasmid pUC119 were used for gene cloning and expression.

DNA techniques

Southern-blot and colony-hybridization experiments were performed with digoxigenin-labeled DNA and a detection kit from Boehringer Mannheim. Single point mutations were introduced into the cloned FDH gene with a DNA mutagenesis kit from Amersham. The DNA sequencing was performed with an Applied Biosystems DNA sequencer 370A and a dye-labeled terminator sequencing kit (Applied Biosystems).

DNA synthesis by the polymerase chain reaction (PCR) was performed with a DNA thermal cycler (Perkin-Elmer) in a 0.1-ml mixture containing 0.02–0.2 mM dNTP 20–100 pmol primers, 10–200 ng template DNA, 0.01 ml 10× reaction buffer, and 2.5 units *Taq* DNA polymerase (Takara). The program of PCR was denaturation by incubation at 95°C for 1 min, annealing at 50–65°C for 2 min, and extension at 72°C for 2 min, for a total 30 cycles.

Protein purification and assays

McFDH from *E. coli* cloned cells was purified essentially in the same manner as described previously (Tishkov et al. 1993): ammonium sulfate fractionation, phenyl-Superose hydrophobic chromatography, and Superdex-200 gel filtration were used. Protein was determined by the method of Bradford.

FDH was assayed by following the reduction of NAD^+ in 0.1 M phosphate buffer (pH 7.0) containing sodium formate as a substrate, with a Shimadzu MPS2000 spectrophotometer at 37°C. Kinetic constants were obtained from duplicate or triplicate measurements of initial rates by varying the concentrations of one substrate at a fixed and saturating concentration of the second.

The thermostability of FDH was examined by incubation of 0.2 mg/ml protein solution in 0.1 M phosphate buffer (pH 7.0) containing 0.01 M EDTA at 60.5°C. Aliquots were withdrawn and residual FDH activity was determined.

Results

Cloning of the McFDH gene

All FDH sequenced so far show high sequence similarity to each other. Therefore we have considered that the amino acid sequences of McFDH and PsFDH are also very close to each other. Two degenerate oligonucleotide primers with sense and antisense sequences corresponding to the regions between amino acid residues 72 and 78 and also between residues 194 and 200 of PsFDH were synthesized: 5′-GTG(or C) ACG(or C) TCG(or C) GAC(or T) AAGGAG(or C) CC-3′ and 3′-CAG(or C) CCG(or C) TGG(or C) CAG(or C) CG(or C) G(or C) CGG(or C) CC-5′.

These regions correspond to the parts of FDH with the highest sequence similarity among all known FDH (Fig. 1). PCR amplification using these primers and a template genomic DNA from *M. vaccae* N10 generated a 400-bp DNA.

The genomic DNA of *M. vaccae* N10 was digested with different restriction endonucleases and subjected to Southern-blot hybridization by means of the DNA generated by PCR and labeled with digoxigenin. A *Hin*dIII-*Pst*I fragment of approximately 2300 bp was selected to be cloned because it was long enough to contain the entire McFDG gene.

The genomic DNA was digested with *Hin*dIII and *Pst*I, and separated by gel electrophoresis in 0.7% low-melting-temperature agarose. The DNA fragments of 2–3 kbp were isolated and ligated into *Hin*dIII-*Pst*I sites of pUC119. About 1000 colonies were examined by colony hybridization with the digoxigenin-labeled PCR DNA. Three colonies showed positive signals. Plasmid DNA isolated from each of these three clones contained the same 2.3-kbp *Hin*dIII-*Pst*I insert and generated the same 400-bp DNA when used as a template in PCR with the same degenerate primers described above. The plasmid DNA coding for the McFDH gene was named pMcFDH. The FDH activity in the extracts of these clone cells cultivated in the presence of isopropyl-β-D-thiogalactoside (IPTG) confirmed the expression of the McFDH gene in *E. coli*.

Determination of the nucleotide sequence

Plasmid pMcFDH was isolated and a preliminary restriction map of the cloned *Hin*dIII-*Pst*I fragment was established with the most commonly used restriction enzymes. In addition to the standard M13 forward and reverse primers, specific primers and a series of deletion clones were prepared and used for sequencing. Both strands were sequenced by the dideoxy-DNA chain-termination method with an Applied Biosystems DNA sequencer. The open-reading frame of 1200 bp encoded a protein with an extremely high sequence similarity to that of PsFDH. The putative protein contained 400 amino acid residues: the same number as that in PsFDH. The two nucleotide sequences differed from each other at 11 positions, and the deduced amino acid sequences were different in two amino acids: isoleucine-36 (threonine in PsFDH) and glutamate-61 (lysine in PsFDH).

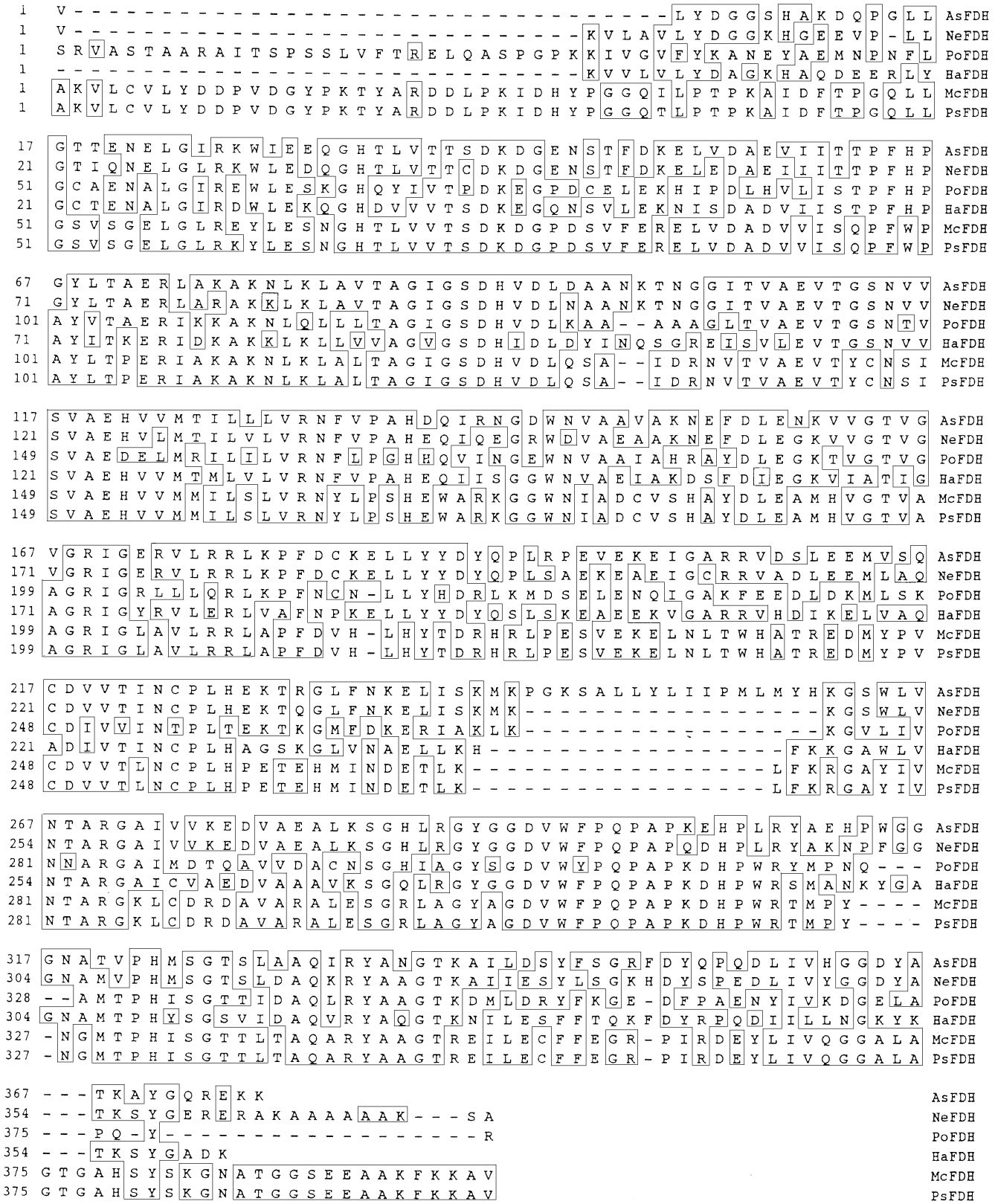

Fig. 1 Alignment of amino acid sequences of various formate dehydrogenases (FDH). *AsFDH* FDH-like protein of *Aspergillus nidulans* (Saleeba et al. 1992); *HaFDH* FDH of *Hansenula* (Hollenberg and Janowicz 1989); *NeFDH* FDH from *Neurospora crassa* (Chow and RajBhandary 1993); *PsFDH* FDH from *Pseudomonas* sp. 101 (Tishkov et al. 1991); *McFDH* FDH from *Mycobacterium vaccae* N10; *PoFDH* FDH from potato (Des Francs-Small et al. 1993). The conserved residues are boxed. The numbers on the left side are the residue numbers of each amino acid sequence

Purification and characterization of the McFDH gene product

The FDH activity in the cell extracts of *E. coli* TG1 harboring pMcFDH was measured. Little activity was found in the extract of the cells cultivated in the absence of IPTG, whereas high activity was observed in the *E. coli* cells cultivated in the presence of IPTG. By comparison of the specific activity of the purified enzyme with that of the cell extract, the amount of McFDH produced in the clone cells was estimated to be about 2% of the total soluble protein. McFDH was purified essentially by the same procedure as that used for PsFDH. The catalytic and physicochemical properties of the two FDH were closely similar to each other: both are dimers of a subunit with the identical molecular mass of 44 000; both show the same k_{cat} value ($10\ s^{-1}$), and similar K_m values for formate (McFDH, 6.0 mM; PsFDH, 6.5 mM) and for NAD^+ (McFDH, 0.09 mM; PsFDH, 0.08 mM). However, the two FDH were distinct from each other in thermostability: PsFDH is more stable than McFDH (Table 1).

Construction and properties of mutant FDH

The amino acid sequences of McFDH and PsFDH differ from each other only at two positions: residues 35 and 61. We attempted to find out the effect of these amino acid residues on thermostability of the enzymes by site-directed mutagenesis. Glutamate-61 of McFDH was replaced by lysine (K), glutamine (Q), and proline (P) with oligonucleotides of the 28-mer: 5′-CCG-TTGGACTCGAGATATG(or T) G (or T) GCGCAG-GC-3′.

The E61K mutant enzyme of McFDH corresponds to the T351 mutant enzyme of PsFDH. The replacement of glutamate-61 by glutamine was expected to give information about the role of the negative charge at position 61 in the stability of McFDH. We also expected the mutant enzyme E61P to become structurally more rigid to improve thermostability.

Table 1 Comparison of kinetic properties and thermostabilities of mutant and wild-type formate dehydrogenases (FDH). The rate constant of the enzyme inactivation at 60.5°C, k_{inact}, was calculated from the averages of two or three independent determinations. *McFDH* FDH from *Mycobacterium vaccae*, *PsFDH* FDH from *Pseudomonas* sp. 101

Enzyme	Specific activity (units/mg)	$10^{-2}\times k_{inact}$ (min^{-1})	K_m (mM)	
			Formate	NAD^+
McFDH	14	3.5	6.0	0.09
McFDH(E61-Q)	12	2.3	8.0	0.11
McFDH(E61-P)	15	1.3	6.5	0.10
McFDH(E61-K)	14	1.4	7.0	0.09
PsFDH	14	1.1	6.5	0.08

Each of all the mutant McFDH was purified to homogeneity in the same manner as the wild-type enzyme with a yeild similar to that for the wild-type enzyme. All enzymes exhibited identical electrophoretic properties upon sodium dodecyl sulfate/polyacrylamide gel electrophoresis (data not shown). The specific activities and K_m values of the mutant enzymes were also very similar to those of the wild-type enzyme (Table 1). This shows that the replacement of glutamate by lysine, proline and glutamine at position 61 rendered the enzyme more thermostable. However, lysine and proline stabilized the enzyme much more effectively than glutamine.

Discussion

The FDH gene of *Mycobacterium vaccae* N10 has been cloned into *E. coli*. The FDH gene from *Pseudomonas* sp. 101 was 99% identical with that from *Mycobacterium vaccae* N10 in nucleotide sequence. The identity in the amino acid sequence was actually 99.5%. Complete and N-terminal amino acid sequences of five and one FDH, respectively, from different sources have been determined so far (Asano et al. 1988; Hollenberg and Janowicz 1989; Chow and RajBhandary 1993; Des Francs-Small et al. 1993). Recently, another protein of unknown function, with a high sequence similarity to FDH, was found in *Aspergillus nidulans* (Saleeba et al. 1992). All FDH as well as this FDH-like fungal protein show extremely high sequence similarity to each other. Thus, FDH have most probably diverged from each other only recently in their evolution.

The deduced amino acid sequence of McFDH differs from that of PsFDH only at two positions: 35 (isoleucine, McFDH; threonine, PsFDH) and 61 (glutamate, McFDH; lysine, PsFDH). The replacement of threonine by isoleucine at position 35 of PsFDH resulted in no significant effect on the thermostability. The results of X-ray crystallographic analysis of PsFDH showed that position 35 occurs in the middle of a featureless loop at positions 27–40 (Lamzin et al. 1994). The results of site-directed mutagenesis are consistent with this particular location of position 35.

The X-ray crystallographic data have shown that aspartate-43 of PsFDH occurs in the vicinity of lysine-61 (Lamzin et al. 1994). The β-carboxyl group of aspartate-43 forms a salt bridge with the ε-amino group of lysine-61, and thereby the structure of PsFDH is stabilized. The tertiary structure of McFDH has not been solved but is probably very similar to that of PsFDH. The negative charge of glutamate-61 is probably repulsed by aspartate-43 in McFDH, and the protein structure is destabilized. The higher thermostability of E61Q mutant McFDH is also consistent with the proposed destabilizing effect of glutamate-61.

Entropic effects contribute to increased thermostability of various proteins (Matthews et al. 1987). The ring structure of the prolyl residue restricts the conformations of this residue much more tightly than other amino acid residues do. The increased numbers of proline residues affect the thermostability of various proteins (Suzuki et al. 1991). In fact, replacement of glutamate-61 of McFDH by proline effectively increased the thermostability of the enzyme to a level similar to that of the E61K mutant enzyme. The results could be explained not only by removal of the negative charge at position 61, but also by a decreased conformational entropy of prolyl residue.

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