A Soluble NADH-Dependent Fumarate Reductase in the Reductive Tricarboxylic Acid Cycle of *Hydrogenobacter thermophilus* TK-6[▽]

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Received 26 May 2008/Accepted 16 August 2008

Fumarate reductase (FRD) is an enzyme that reduces fumarate to succinate. In many organisms, it is bound to the membrane and uses electron donors such as quinol. In this study, an FRD from a thermophilic chemolithoautotrophic bacterium, *Hydrogenobacter thermophilus* TK-6, was purified and characterized. FRD activity using NADH as an electron donor was not detected in the membrane fraction but was found in the soluble fraction. The purified enzyme was demonstrated to be a novel type of FRD, consisting of five subunits. One subunit showed high sequence identity to the catalytic subunits of known FRDs. Although the genes of typical FRDs are assembled in a cluster, the five genes encoding the *H. thermophilus* FRD were distant from each other in the genome. Furthermore, phylogenetic analysis showed that the *H. thermophilus* FRD was located in a distinct position from those of known soluble FRDs. This is the first report of a soluble NADH-dependent FRD in *Bacteria* and of the purification of a FRD that operates in the reductive tricarboxylic acid cycle.

Fumarate reductase (FRD) catalyzes the reduction of one molecule of fumarate to succinate, using two electrons. This enzyme reacts with electron donors such as quinol (50), reduced flavin adenine dinucleotide/reduced flavin mononucleotide (FADH₂/FMNH₂) (15), coenzyme M and coenzyme B (20), or NADH (5, 12). Known FRDs can be divided into two major groups (12). Typical FRDs belong to the first group, are membrane bound, and consist of three or four subunits. A catalytic subunit (FrdA) and an iron-sulfur subunit (FrdB) are attached to the membrane by one or two anchor subunits (FrdC [and FrdD]). FrdA possesses the active center, and FrdB transfers electrons to FrdA from the anchor subunit(s). The amino acid sequences of FrdA and FrdB are highly conserved among diverse species. FrdC and FrdD accept electrons from quinols and transfer them to FrdB. An example of this type of FRD is that of *Escherichia coli*, which is encoded by the *frdABCD* operon (10). This type of FRD functions in fumarate respiration under anaerobic conditions and in the maintenance of redox balance (50).

The second group of FRDs consists of soluble monomeric FRDs, and they possess the conserved catalytic region of FrdA of membrane-bound FRDs (12, 37, 40). Compared with the first group, few organisms are known to possess this type of FRD; these include the FRDs of several *Shewanella* species, *Saccharomyces cerevisiae*, and *Trypanosoma brucei*.

The FRDs of *Shewanella* species have been proposed to oxidize cytochromes and are involved in fumarate respiration (18, 34, 46). The two FRDs of *S. cerevisiae* irreversibly reduce fumarate to succinate, using FADH₂, FMNH₂, or reduced riboflavin as electron donors. These enzymes are suggested to be involved in the maintenance of the intracellular redox balance under anaerobic conditions (8, 15). The genes encoding

two FRDs from the protozoan parasite *T. brucei* were studied and provide the first reports of soluble, NADH-dependent FRDs (5, 12). These proteins are proposed to be involved in the maintenance of cellular redox balance.

The FRD of the methanogenic archaeon *Methanothermobacter thermoautotrophicus* has been purified and characterized and is the sole example, to date, of a multimeric soluble enzyme. This cytoplasmic FRD consists of two subunits that are homologous to FrdA and FrdB of membrane-bound FRDs. This enzyme reacts with coenzyme M and coenzyme B and takes part in 2-oxoglutarate biosynthesis (20).

The reverse reaction of fumarate reduction is catalyzed by succinate dehydrogenase (SDH), a membrane-bound enzyme reacting with electron acceptors such as ubiquinone (10, 32). There are two functions of SDH, to provide fumarate as a substrate in the tricarboxylic acid (TCA) cycle and to transfer electrons to quinone as a member of the respiratory chain (50). Membrane-bound FRDs and SDHs are similar in their amino acid sequences and structures, and in particular, the catalytic subunits are highly conserved (50).

Hydrogenobacter thermophilus is a thermophilic, hydrogen-oxidizing bacterium (47). Phylogenetic analysis of 16S rRNA sequences revealed that the genus *Hydrogenobacter* is located in the deepest branch in the domain *Bacteria* (41), along with members of the same family, *Aquificaceae*. *H. thermophilus* is an obligate chemolithoautotroph that assimilates CO₂ as the sole carbon source through the reductive TCA (RTCA) cycle (48). A number of enzymes in this cycle have been studied in this organism (2, 26, 53). Genome projects for some *Aquificaceae* organisms are in progress, and the complete genome of *Aquifex aeolicus* (13) and the draft genomes of *Hydrogenivirga* sp. strain 128-5-R1-1 and *Hydrogenobaculum* sp. strain Y04AAS1 are now available.

The RTCA cycle is presumed to be an ancestral form of the TCA cycle (2). It was first discovered in a photosynthetic bacterium of the family *Chlorobiaceae* (16) and has been found to operate in many autotrophic members of the *Bacteria* and *Archaea* (4, 22, 23, 24, 45, 48, 51, 52). Recent genome analyses

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[▽] Published ahead of print on 29 August 2008.

revealed that the genes encoding the enzymes of the RTCA cycle are widely distributed (13, 39).

Since FRD is expected to function as a member of the RTCA cycle, FRD activity with reduced methyl viologen (MV) or benzyl viologen (BV) has been used to demonstrate the existence of the RTCA cycle in many organisms (3, 4, 16, 22, 23, 24, 45, 52). To our knowledge, none of the FRDs has been purified from organisms that operate the RTCA cycle. The objectives of this study were to purify FRD from *H. thermophilus* and to reveal its novel characteristics.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. thermophilus* TK-6 (IAM 12695; DSM6534) was cultivated aerobically at 70°C as previously described (47).

Cell disruption. *H. thermophilus* cells were harvested by centrifugation at $6,000 \times g$ for 10 min when the optical density at 540 nm reached approximately 2.5. To prepare the cell extract (CFE), the cells were washed with 20 mM Tris-HCl buffer (pH 8.0) and disrupted by sonication. Cell debris was removed by centrifugation at $100,000 \times g$ for 60 min. The supernatant (designated the CFE) was stored at -80°C until use. To prepare the solubilized membrane fraction, the following steps were performed. *H. thermophilus* cells suspended in 20 mM NaPO_4 buffer (pH 7.2) were disrupted in a French press at $15,000 \text{ lb/in}^2$ (four passages). Cell debris was removed by centrifugation at $10,000 \times g$ for 10 min, and the supernatant was further centrifuged at $100,000 \times g$ for 60 min. The pellets of these two centrifugation processes were suspended in 20 mM NaPO_4 buffer (pH 7.2) and stored at -80°C until use. To solubilize the membranes, 0.04% (vol/vol) Triton X-100 was added, and the mixture was stirred for 60 min at 4°C before being used in enzyme assays.

Measurement of enzymatic activity. FRD activity was determined by measuring the decrease in NADH and the production of succinate. When the decrease in NADH was measured, the reaction mixtures contained 50 mM NaPO_4 buffer (pH 6.5), 20 mM fumarate, 0.2 mM NADH, and the enzyme solution in a total volume of 200 to 500 μl . Assays were carried out at 70°C under an Ar gas phase. After 5 min of preincubation, the reaction was initiated by adding NADH and the enzyme solution. The decrease in NADH was monitored at 340 nm on a Beckman DU 7400 spectrophotometer (Beckman Coulter, Fullerton, CA). To analyze FRD activity with NADPH, 0.2 mM NADPH was added instead of NADH. NAD(P)H concentrations were calculated using an extinction coefficient of $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$. For experiments to determine the K_m values for fumarate and NADH, 0.01 to 20 mM fumarate and 0.03 to 0.2 mM NADH were tested. When the production of succinate was measured, the reaction mixtures contained 50 mM NaPO_4 (pH 6.5), 5 or 20 mM fumarate, 0.5 mM NADH or 5 mM MV, and the enzyme solution in a total volume of 500 μl . To analyze FRD activity with FADH_2 , FMNH₂, BV, and ferredoxins, 0.25 mM FAD, 0.25 mM FMN, 10 mM BV, and 10 μM ferredoxins were used, respectively. Ferredoxins were heterologously expressed and purified as previously described (25, 28). MV, BV, FADH_2 , FMNH₂, and ferredoxins were reduced with 5 or 10 mM dithionite (28). The reaction was initiated by adding dithionite or NADH, and assays were carried out at 70°C under an Ar gas phase. Succinate production was determined by the absorbance at 210 nm, using a TSK-gel OApak-A ion-exclusion column (7.8 mm by 30 cm; Tosoh, Tokyo, Japan). One unit of FRD was defined as the amount of protein that oxidizes 1 μmol of NADH or produces 1 μmol of succinate per minute depending on fumarate. SDH activity was determined by measuring the production of fumarate, using a TSK-gel OApak-A column. The assay was modified from that of Holo and Sirevåg (21). The assay mixture contained 50 mM NaPO_4 (pH 6.5), 5 mM succinate, 0.75 mM triphenyltetrazolium chloride, 0.04 mM phenazine methosulfate, and the enzyme solution in a total volume of 500 μl . To assay the reactivity with NAD, triphenyltetrazolium chloride and phenazine methosulfate were excluded from the assay mixture and 1 mM NAD was added instead. The reaction was initiated by adding the enzyme solution or NAD, and assays were carried out at 70°C under an Ar gas phase.

FRD purification. *H. thermophilus* cells (80 g of wet cells) were harvested and disrupted by sonication. CFE was applied to a DE52 anion-exchange open column (25 mm by 15 cm; Whatman, Brentford, United Kingdom) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 . After elution of the bound proteins with the same buffer containing 1 M NaCl, ammonium sulfate was added to the obtained fraction to 30% saturation. This mixture was applied to a butyl-Toyoparl column (22 mm by 20 cm; Tosoh) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 and 30%-saturated ammonium sulfate. All chromatographic steps except for the DE52 column step were per-

formed using an ÄKTA purifier system (GE Healthcare, Piscataway, NJ). Proteins were eluted with a gradient of 30%- to 0%-saturated ammonium sulfate over 220 ml at a flow rate of 4 ml min^{-1} . The active fractions were dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 and applied to a DEAE-Toyoparl column (22 mm by 20 cm; Tosoh) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 . Proteins were eluted with a gradient of 0 to 1 M NaCl over 60 ml at a flow rate of 4 ml min^{-1} . The active fractions were applied to a ceramic hydroxyapatite type I column (16 mm by 11 cm; Bio-Rad, Hercules, CA) equilibrated with 1 mM KPO_4 buffer (pH 6.8) containing 1 mM MgCl_2 . Proteins were eluted with a gradient of 1 to 400 mM KPO_4 buffer over 90 ml at a flow rate of 3 ml min^{-1} . The active fractions were applied to a MonoQ HR 5/5 column (bed volume, 1 ml; GE Healthcare) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 . Proteins were eluted with a gradient of 0 to 1 M NaCl over 25 ml at a flow rate of 0.5 ml min^{-1} . After ammonium sulfate was added to the active fractions to 30% saturation, this solution was applied to a phenyl Superose HR 5/5 column (bed volume, 1 ml; GE Healthcare) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 and 30%-saturated ammonium sulfate. Proteins were eluted with a gradient of 30%- to 0%-saturated ammonium sulfate over 15 ml at a flow rate of 0.5 ml min^{-1} . The active fractions were designated purified FRD and stored at -80°C until use. For further analysis, the solution was applied to a Toyoparl AF-Blue HC-650M column (bed volume, 6 ml; Tosoh) equilibrated with 20 mM NaPO_4 buffer (pH 6.5) containing 1 mM MgCl_2 . Proteins were eluted with a gradient of 0 to 3 M NaCl over 15 ml at a flow rate of 1 ml min^{-1} .

N-terminal amino acid sequence analysis. The N-terminal amino acid sequences of the five subunits of FRD were determined with Procise 492HT (Applied Biosystems, Foster City, CA) from a blotted membrane (0.2- μm Sequi-Blot polyvinylidene difluoride membrane; Bio-Rad).

Protein assay. Protein concentrations were measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL), with bovine serum albumin as a standard protein.

Gel filtration. For estimation of the molecular mass of the protein, gel filtration was performed using a Superose 6 HR 10/30 column (GE Healthcare) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM MgCl_2 and 150 mM NaCl at a flow rate of 0.5 ml min^{-1} . Chromatography was performed using an ÄKTA purifier system. A gel filtration standard (Bio-Rad) was used as the molecular marker for the calibration. Each measurement of standard or sample was performed in triplicate.

PAGE. *H. thermophilus* FRD solution was analyzed by native polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate-PAGE (SDS-PAGE). For native PAGE, FRD solutions were loaded onto a 7.5% polyacrylamide gel. When necessary, after electrophoresis, the lane containing the enzyme solution was cut off and boiled in loading buffer containing 1% SDS at 100°C for 2 min. After being washed with deionized water, the gel strip was stacked on a 13% polyacrylamide gel and SDS-PAGE was conducted. The proteins were visualized by silver staining or Coomassie brilliant blue staining.

Metal content analysis. The concentration of molybdenum in the purified *H. thermophilus* FRD fraction was determined using inductively coupled plasma mass spectrometry (model SPQ9000; SII NanoTechnology, Tokyo, Japan). The enzyme solution was diluted 30-fold with 1% HNO_3 before analysis.

Construction of phylogenetic trees. Phylogenetic analysis was performed using the deduced amino acid sequences of FRD and SDH catalytic and iron-sulfur subunits from various species. The chosen sequences were those of enzymatically studied FRDs and SDHs and homologous sequences of members of the *Aquificaceae* and organisms operating the RTCA cycle. The sequences of L-aspartate oxidases, enzymes homologous to the catalytic subunits of FRD and SDH (7, 35), were excluded based on the presence of an amino acid corresponding to Glu121, an amino acid strictly conserved only among L-aspartate oxidases (7). Also, phylogenetic analysis of FrdC, the catalytic subunits of molybdenum enzymes, and the G subunit of bacterial complex I was performed. The sequences were aligned using MUSCLE (14). After the gap regions were removed, phylogenetic trees were constructed by the neighbor-joining or maximum likelihood method, using PHYLIP 3.67 (17).

Nucleotide sequence accession numbers. The nucleotide sequences of *frdABCDE* have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under accession numbers AB437311 to AB437315.

RESULTS

Purification of FRD from *H. thermophilus*. Previously, FRD activity with reduced MV as an artificial electron donor was detected in the CFE of *H. thermophilus* (47, 48). This time, in

TABLE 1. Purification of FRD from *H. thermophilus*

Fraction	Total activity ^a (U)	Total protein (mg)	Sp act ^a (U/mg)	Purification factor (fold)	Yield (%)
CFE	1,300	3,800	0.35	1.0	100
Butyl-Toyopearl	600	230	2.6	7.5	46
DEAE-Toyopearl	250	34	7.5	21	19
Hydroxyapatite	110	3.6	30	87	8.5
MonoQ	39	0.77	50	140	3.0
Phenyl Superose	3.5	0.049	71	200	0.27

^a FRD activity was measured using NADH as an electron donor.

addition to MV, FRD assays were performed using NADH or BV as an electron donor. NADH-dependent FRD activity was detected in the CFE. The specific activity was 0.35 U/mg protein, which was slightly higher than the value with MV (0.30 U/mg protein). Also, BV-dependent FRD activity slightly lower than the MV-dependent activity was observed in the CFE (0.27 U/mg protein), and hence, MV was chosen as the electron donor for further experiments. Since many species possess membrane-bound FRDs, the FRD activity in the solubilized membrane fraction was measured. However, only a trace amount of FRD activity was detected, suggesting that the FRD of *H. thermophilus* is localized in the cytosol.

A soluble FRD was purified from the CFE of *H. thermophilus* by following the activities, using NADH or reduced MV as an electron donor. In each purification step, the two activities coincided. This suggested that a single enzyme was able to use both MV and NADH as electron donors. From 80 g of wet cells, 0.049 mg of the purified enzyme was obtained (Table 1).

Subunit composition of purified FRD. Five bands appeared in the SDS-PAGE gel containing the purified enzyme (Fig. 1A). This suggested that the *H. thermophilus* FRD consisted of five subunits, designated FrdABCDE. From the staining intensities of the five bands, the enzyme was presumed to possess equal numbers of each subunit.

In order to confirm that the five bands exist as a complex, the following additional experiments were performed. In native

TABLE 2. The five subunits of *H. thermophilus* FRD

Subunit	GenBank accession no.	N-terminal amino acid sequence	Molecular mass ^a (kDa)	Comments ^a
FrdA	AB437311	MRAYDIVIIG	64	FRD catalytic center (homologous to FrdA of typical membrane-bound FRDs)
FrdB	AB437312	MILRLSIRRE	26	[Fe-S] cluster (homologous to FrdB of typical membrane-bound FRDs)
FrdC	AB437313	MKSTNLPLTV	60	[Fe-S] cluster
FrdD	AB437314	MRVVIVGNP	54	NAD-binding domain
FrdE	AB437315	MRKKPVMIID	21	[Fe-S] cluster

^a Molecular masses and motifs were predicted from the deduced amino acid sequences.

PAGE, these five bands formed a single spot (Fig. 1B), and it was ascertained by subsequent SDS-PAGE that this spot consisted of the five bands. In addition, these five subunits coeluted when the FRD solution was applied to two more columns, namely, Toyopearl AF-Blue HC-650M and Superose 6 HR 10/30 columns. These results provided further evidence that the *H. thermophilus* FRD consists of these five subunits.

The N-terminal amino acid sequence of each band was determined, and the full-length genes were identified based on the genome (unpublished data) of *H. thermophilus* (Table 2). The molecular masses of FrdA, FrdB, FrdC, FrdD, and FrdE calculated from the deduced amino acid sequences were consistent with those from SDS-PAGE (Fig. 1A). The genes encoding the five subunits were inferred to be dispersed in the genome. Genes homologous to all five subunits of the *H. thermophilus* FRD were also found in the complete genome of *A. aeolicus* (13), and they existed apart from each other.

Gel filtration was performed, and the native molecular mass of the enzyme was predicted to be approximately 200 kDa. The enzyme was presumed to be a pentamer consisting of one molecule each of the five subunits, since the calculated molecular mass of the enzyme (225 kDa) (Table 2) matched the above result of gel filtration.

The full-length FrdA and FrdB subunits showed high sequence identity to the known catalytic and iron-sulfur subunits of membrane-bound FRDs, respectively (Tables 2 and 3). The FrdB sequence contained motifs characteristic of one [2Fe-2S] and two [4Fe-4S] clusters. These motifs were found in the B subunits of some archaeal SDHs, while bacterial and other archaeal FRD/SDH B subunits contain one [2Fe-2S], [3Fe-4S], and [4Fe-4S] cluster each (1, 44). FrdD possessed a NAD-binding domain. FrdC and FrdE were each predicted to have one and four [Fe-S] clusters, respectively.

The [Fe-S] cluster motif in FrdC is conserved in the catalytic subunits of molybdenum enzymes and the G subunit of complex I (NuoG) of some bacteria (42), and FrdC was similar in its amino acid sequence to the corresponding regions in these polypeptides. Therefore, it was of interest to determine whether *H. thermophilus* FRD possessed a molybdenum cofactor. Metal content analysis of the purified enzyme indicated that <0.02 molecule of molybdenum was contained per molecule of the enzyme. This result suggested that a molybdenum

FIG. 1. SDS-PAGE (13% acrylamide gel) of purified FRD (A) and native PAGE (7.5% acrylamide gel) of FRD solutions from late purification steps (B). (A) Lane 1, molecular mass markers (each band was 0.40 μ g protein); lane 2, purified FRD (total, 1.5 μ g protein). (B) Lane 1, DEAE fraction; lane 2, hydroxyapatite fraction; lane 3, MonoQ fraction; lane 4, purified FRD. Equivalent amounts of FRD activity were applied for all fractions.

TABLE 3. Motifs in the five subunits of *H. thermophilus* FRD

Subunit	Motif	Position (aa)	Conserved sequence
FrdA	FAD-binding motif ^a	9–15	[I/V]GXGX ₂ G
	Site of covalent FAD attachment ^b	40–49	RSH[T/S]X ₂ AX[G/S]G
FrdB	[2Fe-2S] cluster ^c Two [4Fe-4S] clusters ^d	53–73	CX ₄ CX ₂ CX ₁₁ C
		142–153	CX ₂ CX ₂ CX ₃ CP
		197–208	CX ₂ CX ₂ CX ₃ CP
FrdC	[4Fe-4S] cluster ^e	21–58	[C/H]X _{2–3} CX ₃ CX _{27–34} CX[K/R]
FrdD	NAD-binding motif ^f	165–191	GX _{1–2} GX[I, V, L] GX _{approx 20} [D/E]
FrdE	Four [4Fe-4S] clusters ^g	14–24	CX ₂ CX ₂ CX ₃ C
		59–71	CX ₂ CX ₄ CX ₃ C
		91–101	CX ₂ CX ₂ CX ₃ C
		118–137	CX ₂ CX _{11–15} CX ₃ C

^a Conserved in all FRD/SDH catalytic subunits (11).

^b Conserved in the membrane-bound FRD/SDH A subunits (6) (Fig. 3).

^c Conserved in the membrane-bound FRD/SDH B subunits (1).

^d Found in the B subunits of some archaeal SDHs. Bacterial and other archaeal FRD/SDH B subunits contain one [3Fe-4S] and one [4Fe-4S] cluster instead of two [4Fe-4S] clusters (1, 44).

^e Conserved in the catalytic subunits of molybdenum enzymes and NuoG (42).

^f Conserved in various NAD-binding proteins (9).

^g Conserved in the iron-sulfur subunits of molybdenum enzymes and hydrogenases (27).

cofactor was absent from this enzyme, as well as known NuoG, although the possibility that a molybdenum cofactor was lost during purification steps could not be excluded. FrdE possessed four [Fe-S] cluster motifs, which are also conserved in the iron-sulfur subunits of molybdenum enzymes and hydrogenases (see below).

A phylogenetic tree was constructed using amino acid sequences of the catalytic subunits of FRDs and SDHs (Fig. 2A). A phylogenetic tree of the iron-sulfur subunits of FRDs and SDHs was also constructed, and the topology was almost the same as that for the catalytic subunit (Fig. 2B). As shown in Fig. 2A, the known soluble monomeric FRDs formed a cluster distinct from those of the membrane-bound FRDs, and the soluble FRD of *M. thermoautotrophicus* was located apart from them. The *H. thermophilus* FRD was located in a different position from the other soluble FRDs, suggesting that they are phylogenetically distant. The *H. thermophilus* FRD formed a branch along with the homologs of members of the *Aquificaceae*, and no FRDs or SDHs from other organisms were found that belonged to this branch.

Kinetic characteristics. Kinetic parameters were investigated using the purified enzyme. The optimum temperature for the *H. thermophilus* FRD was 70 to 80°C, which is equal to the growth temperature of this organism. The optimum pH for the reaction was 6.0 to 6.5.

High NADH-dependent FRD activity was detected at low concentrations of fumarate or NADH. The FRD activity in the presence of 50 μ M fumarate was estimated to be 90% of that with 20 mM fumarate. Similarly, the FRD activity in the presence of 40 μ M NADH was estimated to be 70% of that with 0.2 mM NADH (data not shown). In other organisms, the K_m values for fumarate were reported to vary from 0.005 to 3 mM

(10, 19, 29, 33, 36, 38). Although the K_m values for fumarate and NADH were too low to be determined accurately, the affinities for fumarate ($K_m < 50 \mu$ M) and NADH ($K_m < 40 \mu$ M) were considered to be high. Therefore, the enzyme was regarded to possess the characteristics required to work sufficiently as a NADH-dependent FRD in vivo. When NADPH, FADH₂, or FMNH₂ was used as an electron donor, no significant activity was detected. Also, several enzymes in the RTCA cycle are known to react with ferredoxins, and several ferredoxins have been found in *H. thermophilus* (25, 28). Therefore, the reactivity with these ferredoxins was examined, but no ferredoxin-dependent FRD activity was detected.

The specific activity of the MV-dependent FRD reaction was 33 U/mg protein. This value was 47% of the NADH-dependent activity. A wide range of specific FRD activities (0.03 to 310 U/mg protein) have been reported for other organisms (19, 20, 29, 31, 36).

The enzyme activity in the reverse direction, the SDH activity, was reported for the CFE of *H. thermophilus* (47). The SDH activities of CFE and the purified enzyme were assayed using NAD as an electron acceptor, and significant activity was not detected in either sample. This was consistent with the redox potentials of the substrates involved in the reaction (−320 mV for NAD/NADH and +30 mV for fumarate/succinate) (29). Thus, it was assumed that this enzyme preferentially catalyzes the reaction in the direction of fumarate reduction when NAD/NADH is used as the electron acceptor/donor. Further assays were performed with highly purified enzyme fractions, using triphenyltetrazolium chloride and phenazine methosulfate, a common method for measuring SDH activity, and no activity was detected (<1% of the FRD activity).

DISCUSSION

This report provides, to the best of our knowledge, the first example of a soluble, NADH-dependent FRD in the domain *Bacteria* and also of the purification of FRD from an RTCA cycle-operating organism.

FRDs are generally membrane-bound and consist of three or four subunits. Most of the known bacterial FRDs directly oxidize quinols for ATP synthesis (10, 30). Therefore, it is essential for these enzymes to be located in the membrane. In contrast, the *H. thermophilus* FRD works as a member of the RTCA cycle. This functional difference might allow the enzyme to be soluble and to react with NADH. On the other hand, some FRDs are known to be soluble and composed of one or two kinds of subunits. The FRDs of several *Shewanella* species, *S. cerevisiae*, *M. thermoautotrophicus*, and *T. brucei* belong to this group. These enzymes are different from the *H. thermophilus* FRD in their functions and, in some cases, their electron donors (5, 8, 12, 15, 18, 20, 46).

In the A subunit of membrane-bound FRDs and SDHs, a histidine residue (His44 in *E. coli* FrdA), which is the binding residue of covalently bound FAD, is highly conserved (6, 37). It is also conserved in *H. thermophilus* FrdA, although it is not conserved in other soluble FRDs (Fig. 3; Table 3). Also, the “GG doublet,” proposed as a conserved motif in FAD-binding domains of known soluble monomeric FRDs (49), was not conserved in *H. thermophilus* FrdA. Along with the phylogenetic tree (Fig. 2A), these findings further support the specu-

FIG. 2. Phylogenetic trees of FRD/SDH catalytic subunits (A) and iron-sulfur subunits (B). The numbers at the nodes are bootstrap confidence values expressed as percentages of 1,000 bootstrap replications. The order of divergence was presumed to be reliable only when the bootstrap values were above 80. The scale bars represent 0.2 estimated change per nucleotide. The trees were constructed by using the neighbor-joining method and showed the same overall topology as the trees constructed by the maximum likelihood method. The organism, gene, and locus tag in the NCBI protein database (in parentheses) for the sequences used in these figures are as follows: for panel A, *Acidianus ambivalens* *sdhA* (CAA06780), *A. aeolicus* *aq_594* (NP_213415), *Bacillus subtilis* *sdhA* (P08065), *Campylobacter jejuni* *frdA* (NP_281599), *E. coli* *frdA* and *sdhA* (*Escherichia* 1 and 2; AAC77114 and AAC73817, respectively), *Geobacter sulfurreducens* *frdA* (NP_952230), *Helicobacter pylori* *frdA* (NP_222899), *Hydrogenovirga* sp. strain 128-5-R1-1 HG1285_17779 (ZP_02177006), *H. thermophilus* *frdA* (AB437311), *Hydrogenobaculum* sp. strain Y04AAS1 HY04AAS1DRAFT_0051 (ZP_02060827), *Magnetococcus* sp. strain MC-1 Mmc1_1746 (YP_865660), *M. thermoautotrophicus* *tfrA* (CAA04398), *Nitriruptor* sp. strain SB155-2 NIS_0006 (YP_001355481), *Paenibacillus macerans* *sdhA* (CAA69872), *Pyrobaculum islandicum* Pisl_0247 (ABL87427), *S. cerevisiae* *FRDS*, *OSM1*, and *SDH1* (*Saccharomyces* 1, 2, and 3; AAB64995, AAB59346, and AAA35026, respectively), *Shewanella frigidimarina* *fccA* (P0C278), *Sulfolobus acidocaldarius* *sdhA* (AAY80343), *Sulfrimonas denitrificans* Suden_0037 (YP_392553), *Sulfurovum* sp. strain NBC37-1 SUN_0152 (YP_001357469), *Thermoproteus neutrophilus* Tneu_0423 (ACB39371), *T. brucei* *frdg* and *frdm1* (*Trypanosoma* 1 and 2; AAN40014 and AAX20163, respectively), and *Wolinella succinogenes* *frdA* (P17412); for panel B, *A. ambivalens* *sdhB* (CAA06781), *A. aeolicus* *aq_553* (NP_213388), *B. subtilis* *sdhB* (P08066), *C. jejuni* *frdB* (NP_281600), *E. coli* *frdB* and *sdhB* (*Escherichia* 1 and 2; AAC77113 and AAC73818, respectively), *G. sulfurreducens* *frdB* (NP_952231), *H. thermophilus* *frdB* (AB437312), *M. thermoautotrophicus* *tfrB* (CAA04399), *Nitriruptor* sp. strain SB155-2 NIS_0010 (YP_001355485), *P. macerans* *sdhB* (CAA69873), *P. islandicum* Pisl_0246 (ABL87426), *S. cerevisiae* *SDH2* (AAS56515), *S. acidocaldarius* *sdhB* (AAY80342), *S. denitrificans* Suden_0038 (YP_392554), *T. neutrophilus* Tneu_0424 (ACB39372), and *W. succinogenes* *frdB* (P17596).

Ht_frdA	AVVSKVYPTRS-HTGAAQGGVNAA
Ec_frdA	ALISKVYPMRS-HTVAAEGG-SAA
Ws_frdA	IVLSLIPVKRS-HSAAQGGMQAS
Ec_sdhA	ALLSKVFPTRS-HTVSAQGGITVA
Sc_SDH1	ACISKLFPTRS-HTVAAQGGINAA
Sf_fcc3	ILIEKEPVIIGG-NAKLAAGGMNAA
Sc_FRDS	TILEKASSIGG-NSIKASSGINGA
Sc_OSM1	VLLDKAASIGG-NSIKASSGINGA
Tb_frdg	VLMEKEAKLGG-NSAKATSGINGW
Tb_frdm1	ILMEKEGRIGG-NSAKATSGINGW
Mt_tfrA	IIVSKGLSEKSGCTGMAEGGYNAA

FIG. 3. Alignment of amino acid sequences of catalytic subunits. The organism and gene for the sequences used in this figure are as follows: Ht, *H. thermophilus* *frdA*; Ec, *E. coli* *frdA* and *sdhA*; Ws, *Wolinella succinogenes* *frdA*; Sc, *S. cerevisiae* *SDH1*, *FRDS*, and *OSM1*; Sf, *Shewanella frigidimarina* *fccA*; Tb, *T. brucei* *frdg* and *frdm1*; Mt, *M. thermoautotrophicus* *tfrA*. The conserved histidine (His42 in *H. thermophilus*) and the "GG doublet" (49) are shown with a gray and a white box, respectively.

lation that the *H. thermophilus* FRD is a different type of FRD from the known soluble FRDs.

In this study, it was found that the *H. thermophilus* FRD has a subunit composition different from that of either group of FRDs. This enzyme consisted of five subunits, and unlike membrane-bound FRDs, the genes corresponding to these subunits were not grouped in an operon but were interspersed throughout the genome. Two of the five subunits were homologous to subunits A and B of typical membrane-bound FRDs, but none were homologous to the anchor subunits of membrane-bound FRDs.

Because of its novel subunit composition, the functions of the five subunits of the *H. thermophilus* FRD are of interest. Since *FrdA* and *FrdB* of this enzyme have high sequence iden-

FIG. 4. Phylogenetic tree of *H. thermophilus* FrdC and its homologous sequences in members of the *Aquificaceae*, with the catalytic subunits of molybdenum enzymes and NuoG. The numbers at the nodes are bootstrap confidence values expressed as percentages of 1,000 bootstrap replications. The order of divergence was presumed to be reliable only when the bootstrap values were above 80. The scale bar represents 0.5 estimated change per nucleotide. The tree was constructed by using the neighbor-joining method and showed the same overall topology as the tree constructed by the maximum likelihood method. The organism, gene, and locus tag in the NCBI protein database (in parentheses) for the sequences used in this figure are as follows: *A. aeolicus* aq_116 (NP_213078), *Buchnera aphidicola* subsp. *Baizongia pistaciae* nuoG (NP_777777), *Escherichia coli* K-12 strain MG1655 *dmsA*, *fdhF*, *fdnG*, and *narG* and *Escherichia coli* O157:H7 nuoG (Escherichia 1, 2, 3, 4, 5; AAC73980, AAD13462, AAD13438, AAC74308, and Q8XCX2, respectively), *Haloarcula marismortui* narG (CAB89111), *Haloferax mediterranei* narG and *nasA* (*Haloferax* 1 and 2; CAF21906 and CAF19042, respectively), *Hydrogenivirga* sp. strain 128-5-R1-1 HG1285_08874 (ZP_02178505), *H. thermophilus* frdC (AB437313), *Hydrogenobaculum* sp. strain Y04AAS1 HY04AAS1DRAFT_0487 (ZP_02060585), *Klebsiella pneumoniae* nasA (B49847), *Methanobacterium formicicum* fdhA (J02581), *Mycobacterium tuberculosis* nuoG (NP_337764), *Pseudomonas aeruginosa* nuoG and *Pseudomonas putida* narB (*Pseudomonas* 1 and 2; AAG06030 and AAF74559, respectively), *Rhodobacter capsulatus* dorA and *nasA* (*Rhodobacter* 1 and 2; Q52675 and AAQ18186, respectively), *Shewanella oneidensis* nuoG (NP_716644), *Sinorhizobium meliloti* nuoG2 (AAK65486), *Streptomyces coelicolor* nuoG (NP_628730), and *Thermus thermophilus* nqo3 (Q56223). DMSO, dimethyl sulfoxide.

tity to those of the membrane-bound FRDs, they might have similar functions. The catalytic center of the *H. thermophilus* FRD is most likely in FrdA, as in the case of other FRDs. FrdD possesses a NAD-binding domain and therefore may enable the enzyme to react with NADH.

The functional roles of FrdC and FrdE are currently unclear. The FrdC sequence showed relatively low similarities to the catalytic subunits of molybdenum enzymes and the C-terminal region of NuoG. In the catalytic subunits of molybdenum enzymes, such as respiratory and assimilatory nitrate reductase, formate dehydrogenase, and dimethyl sulfoxide reductase, the conserved cysteine motif (Table 3) coordinates a [4Fe-4S] cluster and a molybdenum cofactor (27, 42). In contrast, NuoG neither contains a molybdenum cofactor nor functions as a catalytic subunit, and the role of the [4Fe-4S] cluster in its C-terminal region remains unclear (43). To further investigate whether FrdC is phylogenetically closer to molybdenum enzymes or NuoG, a phylogenetic tree of these sequences was constructed (Fig. 4). *H. thermophilus* FrdC and its homologs in members of the *Aquificaceae* did not belong to either group significantly due to the low bootstrap value. Considering the absence of molybdenum in the purified enzyme and the fact that FrdA is highly similar in its sequence to the catalytic subunits of known FRDs, it is conceivable that FrdC is not a catalytic subunit and might have an unknown function. FrdE was homologous in its amino acid sequence to the proteins that bind four [Fe-S] clusters, including the iron-sulfur subunits of molybdenum enzymes and hydrogenases (e.g., 39% and 32% sequence identity with the dimethyl sulfoxide reductase β subunit [GenBank accession no. AAC73980] and the hydrogenase

3 iron-sulfur subunit [GenBank accession no. AAC75766] of *E. coli*, respectively). These proteins possess four [Fe-S] clusters ligated by 16 conserved cysteine residues (27) (Table 3). Although the function of FrdE has not been identified yet, it might function as an electron-accepting/donating component in the oxidation-reduction pathway.

In the phylogenetic tree of FrdA homologs (Fig. 2A), other members of the *Aquificaceae* (*A. aeolicus*, *Hydrogenivirga* sp., and *Hydrogenobaculum* sp.) possessed homologs that branched near the *H. thermophilus* FRD, occupying a different position from that of known soluble FRDs. The FrdB and FrdC homologs of these organisms also branched near the *H. thermophilus* FRD in the phylogenetic trees (Fig. 2B and 4). These were the only organisms that contained genes with high sequence identities to the whole lengths of *frdC* and *frdD*. In addition, the genomes of these three organisms each contained open reading frames homologous to all five subunits of the *H. thermophilus* FRD. It is speculated that these organisms also possess FRDs that are composed of five subunits, homologous to that of *H. thermophilus*.

As mentioned above, the genes encoding the A subunits of membrane-bound FRDs generally form a cluster with the genes for other subunits in their genomes. However, genes encoding FrdA homologs that do not form a cluster with the genes of anchor proteins were found in the genomes of some organisms known to possess the RTCA cycle, such as *Magnetococcus* sp. strain MC-1, *Nitratiruptor* sp. strain SB155-2 (39), *Pyrobaculum islandicum*, *Sulfurimonas denitrificans*, *Sulfurovum* sp. strain NBC37-1 (39), and *Thermoproteus neutrophilus*. It is interesting to question whether soluble FRDs are present

in these organisms. It was not possible to speculate about what kind of FRDs they have based on the phylogenetic tree (Fig. 2A) because the FrdA homologs of these organisms were not distinctly located within the clusters of known FRDs. Since FRD activities were assayed only by using MV or BV in these organisms (4, 22, 24, 45, 52), there is a possibility that FRD activities with NADH or other types of reductants have been overlooked. Furthermore, there are reports of FRD activity in the cytoplasmic fractions of *P. islandicum* (22) and *T. neutrophilus* (45). These organisms operating the RTCA cycle may possess FRDs that are not typical membrane-bound enzymes.

The intriguing discovery of a five-subunit FRD raised important questions about the role of each subunit, especially the subunits FrdC, FrdD, and FrdE. Moreover, the reaction mechanism of this enzyme is of great interest because it possesses many domains that may be involved in redox reactions, such as a FAD-binding domain, [Fe-S] clusters, and a NAD-binding domain. Further studies are being conducted in order to answer these questions.

ACKNOWLEDGMENTS

This research was supported in part by grants-in-aid for scientific research from the Japan Society for the Promotion of Science.

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