**Molybdenum-containing membrane-bound formate dehydrogenase isolated from
Citrobacter sp. S-77 having high stability against oxygen, pH, and temperature**

Nga T. Nguyen,¹ Takeshi Yatabe,² Ki-Seok Yoon,^{1,2} Seiji Ogo^{1,2,3*}

Department of Chemistry and Biochemistry, Graduate School of Engineering, Kyushu University, 744 Moto-oka, Nishi-ku, Fukuoka 819-0395, Japan,¹ International Institute for Carbon-Neutral Energy Research (WPI-I2CNER), Kyushu University, 744 Moto-oka, Nishi-ku, Fukuoka 819-0395, Japan,² and Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Kawaguchi Center Building, 4-1-8 Honcho, Kawaguchi-shi, Saitama 332-0012, Japan³

* Corresponding author: International Institute for Carbon-Neutral Energy Research (WPI-I2CNER), Kyushu University, 744 Moto-oka, Nishi-ku, Fukuoka 819-0395, Japan
Tel.: +81 92 802 6688; fax: +81 92 802 6688.

E-mail address: ogotcm@mail.cstm.kyushu-u.ac.jp (S. Ogo).

Key words:

Molybdenum-containing formate dehydrogenase; *Citrobacter* sp. S-77; formate oxidation; O₂-stability; thermostability; pH resistance

Running Title: Formate dehydrogenase from *Citrobacter* sp. S-77

ABSTRACT

Membrane-bound formate dehydrogenase (FDH) was purified to homogeneity from a facultative anaerobic bacterium *Citrobacter* sp. S-77. The FDH from *Citrobacter* sp. S-77 (FDH_{S77}) was a monomer with molecular mass of approximately 150 kDa. On SDS-PAGE, the purified FDH_{S77} showed as three different protein bands with molecular mass of approximately 95, 87, and 32 kDa, respectively. Based on the N-terminal amino acid sequence analysis, the sequence alignments observed for the 87 kDa protein band were identical to that of the large subunit of 95 kDa, indicating that the purified FDH_{S77} consisted of two subunits; a 95 kDa large subunit and a 32 kDa small subunit. The purified FDH_{S77} in this purification did not contain a heme *b* subunit, but the FDH_{S77} showed significant activity for formate oxidation, determined by the $V_{\max}$ of 30.4 U/mg using benzyl viologen as an electron acceptor. The EPR and ICP-MS spectra indicate that the FDH_{S77} is a molybdenum-containing enzyme, displaying a remarkable O₂-stability along with thermostability and pH resistance. This is the first report of the purification and characterization of a FDH from *Citrobacter* species.

INTRODUCTION

Biological carbon dioxide conversion is extremely important for all organisms, being very closely linked to the flow of energy that is maintained by living organisms in the biosphere. Organisms that use CO₂ as a carbon source may be employed to produce useful compounds that relate to the biological production of energy from redox or electron transport proteins, such as photosynthetic reaction centers, hydrogenase, formate dehydrogenase (FDH), pyruvate ferredoxin oxidoreductase, CO dehydrogenase, etc. (1-3). Among them, FDH is one of the most attractive metalloenzymes that catalyzes the reversible oxidation of formate to produce two-electrons and CO₂, playing an important role in the aerobic and anaerobic oxidative metabolism (2-4). Despite the importance of this process in nature, many key aspects of the biochemistry and molecular mechanism of the enzyme are still unclear (2,4,5). FDH also has a great potential for use as a biocatalyst in biotechnological applications, such as for the regeneration of cofactors of NAD(P)H in the pharmaceutical industry (6) and for an effective homogenous, formate-oxidizing biocatalyst in safe fuel cell systems. Additionally, FDH has a great potential as an effective biocatalyst for conversion of CO₂ to make formate as an alternative fuel (7-12). Compared to H₂, formate is a favourable energy carrier as a non-flammable fuel (2,8), enabling it to be easily stored and safely transported. In fact, the FDH has served as an effective biocatalyst for electron donors in an energy supplying system and for reduction of CO₂ to formate (10-12). However, the enzyme is very sensitive to O₂, easily losing its catalytic activity after air oxidation (13,14). The O₂-sensitivity of the FDH is considered a major bottleneck to practical use of the enzyme for biotechnological applications. So far, many FDHs have been purified from various organisms (4,5,13-17). Most FDHs contain metal cofactors of molybdenum or tungsten in their active sites (2,5,9,15-18). Among them, the FDH-N from *Escherichia coli*, which is analogous to the FDH purified from *Citrobacter* sp. S-77 (FDH_{S77}), was purified and characterized (13,18). The crystal structure of

the FDH-N was determined at 1.6 Å, containing a selenium atom that is coordinated to molybdenum in the molybdopterin (MPT) moieties in the active site (5,9,18). The FDH-N from *E. coli* is highly stable under anaerobic conditions, but the enzyme is very sensitive to aerobic conditions (13).

During the course of exploring novel biocatalysts, we have found an O₂-stable membrane-bound FDH from the recently isolated bacterium, *Citrobacter* sp. strain S-77. Although the FDH activity was measured in the cell extracts of *C. amalonaticus* (19) and *C. freundii* (20), the purification and characterization of FDH from the bacteria, however, has not been reported. Here, we present the first purification and characterization of a molybdenum containing FDH from *Citrobacter* species. The strain S-77 is a facultative anaerobic bacterium that grows rapidly to high densities under aerobic and anaerobic conditions (21). In the previous studies, we have purified and characterized a novel membrane-bound [NiFe]hydrogenase (MBH) from the same bacterium (21), which has remarkable O₂-stability and high H₂-oxidation. Like the MBH from *Citrobacter* sp. S-77, the purified FDH_{S77} displays a remarkable O₂-stability, along with thermostability and a wide range of pH resistance. This newly found FDH_{S77} might be an important enzyme that may significantly contribute to the development of effective biocatalysts for CO₂ conversion.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Citrobacter sp. S-77 was grown in 20 L Carboy bottles containing a 15 L growth medium. The medium was modified as previously described (21) supplemented with the following components: 3.0 g yeast extract, 3.0 g peptone, 0.5 g MgSO₄·7H₂O, 3.0 g (NH₄)₂SO₄, 2.0 g K₂HPO₄, 1.0 g KH₂PO₄, 1.0 g D-glucose, 2.0 g sodium formate, 0.2 g ammonium iron(III) citrate, and 0.1 g of CaCl₂ and the minor mineral components of 0.1 M Na₂WO₄, 0.1 M

Na₂MoO₄, and 0.2 M Na₂Se₂O₃. The strain S-77 easily entered the stationary phase after 2 days of culture at 32°C. The cells were harvested by centrifugation at 9,000 ×g for 20 min. The precipitated pellet was frozen in liquid N₂ and stored at -80°C.

Membrane preparation and solubilization of FDH from the membrane

Frozen cells (70.0 g) were suspended in 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.0) and disrupted by sonication four times for 3 min in an ice bath at 60 W with an Ultrasonic Disruptor UD-200 (TOMY SEIKO Inc., Japan). The broken cells were centrifuged at 3,000 ×g for 20 min to remove cell debris and the membrane, was precipitated by ultracentrifugation (Optima L-90K, Beckman Coulter Inc., USA) at 150,000 ×g for 1 h at 4°C. The membrane was washed once with the same buffer containing 1 M NaCl to remove cytoplasmic contaminants bound in the membrane. The washed membranes were suspended to be 4 mg/mL protein concentration in 20 mM MOPS (pH 7.0) containing 1.0 M NaCl and 1 mM dithiothreitol (DTT). FDH was solubilized by adding 0.5% Sulfobetaine 3-12 (SB3-12) as a zwitterionic detergent. Immediately after addition of the detergent, the membrane solution was slowly stirred at 4°C for 3 h under 100% N₂ gas. The extract was then centrifuged at 150,000 ×g for 1 h. The supernatant was used for purification of FDH.

Purification of FDH from *Citrobacter* sp. S-77

All purification procedures were carried out at room temperature under strictly anaerobic conditions using a Coy anaerobic chamber under an atmosphere of 98% N₂ and 2% H₂. All buffers were repeatedly degassed and flushed with N₂ and were stored in the anaerobic chamber. Protein purification was performed in the Coy anaerobic chamber using an AKTA-FPLC system (GE Healthcare). The solubilized membrane proteins were directly loaded onto hydroxyapatite (2.5 × 15 cm; Bio-Rad Laboratories Inc.), pre-equilibrated with 1

mM potassium phosphate (KP) buffer (pH 7.0) containing 0.5 mM DTT and 0.2% SB3-12 at a flow rate of 8.0 mL/min. FDH activity was detected around 50 mM concentration of KP by a linear gradient between 1-100 mM KP buffer. The active pools of FDH were directly applied onto a Q Sepharose HP (1.6 × 12 cm; GE Healthcare, UK) pre-equilibrated with 20 mM Tris buffer (pH 8.0) containing 1 mM DTT and 0.2% SB3-12. After loading the sample, the column was washed with the same buffer and the protein was eluted with a gradient of 0.1-0.35 M NaCl at a flow rate of 4.5 mL/min. The FDH eluted at approximately 0.3 M NaCl was diluted 3-fold with nonsalt buffer and applied onto a Resource 15 Q (0.64 × 3 cm; GE Healthcare, UK). After sample application, the column was washed with wash buffer and then eluted isocratically using a buffer containing 0.2 M NaCl, followed by a gradient of buffer containing 0.25 to 0.4 M NaCl at a flow rate of 1.5 mL/min. At this stage, the FDH eluted at approximately 0.32 M NaCl was concentrated using Amicon Ultra-15 (30,000 NMWL; Millipore Corp., Billerica, MA) and then loaded onto a Superdex 200 column (1.6 × 50 cm; GE Healthcare, UK) pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 0.3 M NaCl and 0.2% SB3-12 at a flow rate of 1.0 mL/min, eluting as a single peak with a molecular weight of approximately 150 kDa. Protein purity was established by SDS-PAGE and Native-PAGE analysis using a 5-20% gradient gel. Activity staining in the nondenaturing gel was carried out in the presence of 10 mM formate and 1 mM triphenyl tetrazolium chloride as an electron acceptor in 50 mM MOPS buffer (pH 7.0) at 30°C under N₂ gas.

Enzyme assays

FDH activity was routinely determined spectrophotometrically by following the formate-dependent reduction of benzyl viologen (BV) at 30 °C in glass cuvettes sealed with a rubber stopper and an aluminum cap under strictly anaerobic conditions. [In order to maintain strictly anaerobic conditions for enzyme assay, all assays were performed under strictly](#)

anaerobic conditions by flushing with a constant flow of N₂ for 5 min using a vacuum gas manifold. The standard assay mixture contained 10 mM BV in 50 mM MOPS buffer, pH 7.0. Activity was calculated by the increase in absorbance of BV at 600 nm ($\Delta_{600\text{ nm}} = 8.3\text{ mM}^{-1}\text{ cm}^{-1}$) (22). The maximal kinetic velocity (V_{max}) and K_m values were determined by fitting the data to the Michaelis-Menten equation using nonlinear regression. The data were analyzed by using the Enzyme Kinetics Module1.1 of SigmaPlot 8.0 software (Jandel Scientific, CA). Activity was expressed as units/mg of protein, where one unit (U) is equivalent to oxidation of 1 μmol formate/min.

Oxygen stability of the purified FDH_{S77}

In order to evaluate O₂-stability of the purified FDH_{S77}, the formate-oxidation activity of the air oxidized enzyme after exposure to air was assayed. The vials containing the purified enzyme solution were flushed with dry air for 5 min and then the enzyme was oxidized by incubation under O₂ at 4°C. The remaining activity of the oxidized enzyme was measured periodically. The atmosphere in the assay cuvettes sealed with a gas-tight rubber stoppers and an aluminum caps was exchanged by flushing with a constant flow of N₂ for 5 min to remove the dissolved trace O₂ in the reaction solution and then the assay was initiated by injection of the enzyme solution.

Influence of pH on FDH activity

For the optimum reaction pH measurement, the Britton-Robinson (BR) universal buffer (pH 5.0–9.0) was used. The optimum pH for the reaction was determined by measuring the activity using a 50 mM Britton–Robinson (BR) buffer system. For the pH stability, the purified FDH_{S77} was incubated in the same BR buffer over a wide pH range (3.0–10.0) at 4°C

under N₂. The pH stability of the enzyme was determined by measuring the residual activity after incubation for 3 h.

Thermal properties of the FDH_{S77}

The optimum reaction temperature was determined by measuring the formate oxidation activity over a temperature range of 20–100°C. Before injection of the enzyme solution, the reaction mixture containing 10 mM BV was pre-incubated for 10 min under N₂ at each measuring temperature. For the thermostability of the purified FDH_{S77}, the enzyme solution (0.1 mg/mL) in a 10 mM Tris-HCl buffer (pH 8.0) containing 0.3 M NaCl and 0.2% SB3-12 was incubated between 0 and 80°C for 20 min under N₂. The denatured protein was then removed by centrifugation at 14,000 ×g for 20 min and subjected to the enzyme assay. The thermostability was determined by measuring the remaining activity after thermal incubation.

Electrochemical experiments

The amperometric *i-t* curve measurement was used to determine O₂-stability of the FDH_{S77}. The amperometric *i-t* curve was performed with a computer controlled electrochemical analyzer (CH Instruments model 760 DT, BAS Inc., Japan) connected to a rotating ring/disk electrode device (RRDE-3, BAS Inc.). An Ag/AgCl/3.0 M NaCl electrode (assumed + 0.197 V vs. SHE; model RE-1B, BAS Inc.) was used as a reference electrode. A platinum wire was used as a counter electrode. A glassy carbon disk working electrode (3 mm in diameter, BAS Inc.) was carefully polished with 0.05 µm alumina/water slurry on a glass-plate mounted microcloth pad and the polished electrode was rinsed with distilled water. Electrochemical measurements were carried out at 30°C by purging the electrolysis cell with N₂ gas or dry air. The reaction mixture (10 mL) contained 200 µg of the purified enzyme, 20 mM formate, 2 mM BV, and 0.2 M NaCl in 50 mM Tris-HCl buffer (pH 8.0). The

temperature of the reaction chamber was controlled by a water jacket.

EPR and UV-visible spectra

X-band electron paramagnetic resonance (EPR) spectra were measured by a JEOL JES-FA200 spectrometer. The air-oxidized and formate-reduced samples were injected into 3-mm-diameter quartz EPR tubes and frozen by slowly immersing the tubes into liquid nitrogen. The formate-reduced enzyme was prepared by promoting the FDH reaction under N₂ for 20 min at 30°C, and then injected into EPR tubes sealed with a rubber stopper. EPR spectra were measured at 110 K under liquid nitrogen. UV-visible spectra were measured using a JASCO spectrophotometer (Jasco, Tokyo, Japan).

Other methods

The N-terminal amino acid sequence of each subunit of the purified enzyme was determined by the automated Edman degradation system of ABI protein sequencer 473A (Applied Biosystems Japan, Tokyo). Each subunit of the enzyme was separated by SDS-PAGE and then blotted onto a polyvinylidene difluoride membrane (23). The protein concentration was measured using established procedures for the Bio-Rad DC Protein Assay (Bio-Rad Laboratories Inc.) (24). The Mo, W, Se, and Fe contents of the purified enzyme were analyzed by an inductively coupled plasma mass spectrometer (ICP-MS) using an Agilent 7500c (Agilent Technologies, Inc., USA). The molecular weight of the purified enzyme was determined by gel filtration using a Superose 12 column calibrated with standard molecular weight markers (Bio-Rad Laboratories Inc.).

RESULTS

Purification of FDH from *Citrobacter* sp. S-77

We could detect a significant FDH activity from the isolated membrane fraction of *Citrobacter* sp. strain S-77. The FDH_{S77} could be effectively solubilized by the zwitterionic detergent of 0.5% SB3-12, which could be recovered to approximately 87% of total FDH activity from the membrane fraction of the strain S-77. In the first column of hydroxyapatite, the FDH_{S77} could be effectively separated from the majority of hydrogenase activity. Through subsequent column chromatographies of Q Sepharose high performance, Resource 15 Q, and Superdex 200, the FDH_{S77} was purified to electrophoretic homogeneity. The purified FDH_{S77} showed a specific activity of 24.5 U/mg for the condition of 10 mM BV. The purified FDH_{S77} on SDS-PAGE gel appeared as three distinct protein bands, estimated to be approximately 95, 87, and 32 kDa, respectively (Fig. 1A). However, according to the results of N-terminal amino acid sequence analysis, the initial amino terminal sequence of the 95 kDa subunit is identical to that of the 87 kDa polypeptide, suggesting that the 87 kDa protein is a degraded product of the large subunit. As shown in Fig. 1B, the initial 19 amino acid residues determined for the FDH_{S77} is the same for that of the FDH-N from *E. coli*, but not that of the FDH-O (Fig. 1B). In addition, the purified FDH_{S77} gave a single protein band on a nondenaturing gel, which was responsible for the activity band of FDH_{S77} (Fig. 1C). Subsequent experimental results showed that the purified FDH_{S77} exhibited a single UV-absorption peak of the gel filtration column of Superdex 200 (Fig. 1D). The molecular weight of the native enzyme, determined by Superose 12 gel filtration was estimated to be approximately 150 ± 20 kDa (Fig. 1E). Taken together, we have concluded that the 87 kDa polypeptide is a degradation byproduct of the 95 kDa polypeptide of large subunit. The large subunit of FDH_{S77} appears to be easily cleaved in the C-terminal region during the step of purification or the development of SDS-PAGE analysis. Based on amino acid comparison of other bacterial FDHs, the sequences of FDH_{S77} have high homology with those of FDH-N from *E. coli*, suggesting that operon structure of the FDH genes of *Citrobacter* sp. S-77 may encode in the *fdnGHI* operon (5). The

operon of FDH-N from *E. coli* encodes three different polypeptides of approximately 110, 32, and 20 kDa (13).

Influence of pH and temperature on FDH activity

The optimal temperature for the FDH_{S77} activity was 80°C (Fig. 2A). The activity at 80°C was almost 5-fold higher than that at 30°C (24.5 U/mg), reaching 132.3 U/mg. Regarding the growth temperature (32°C) of strain S-77, the FDH_{S77} showed a high level of thermostability, indicating a half-life of activity of ca. 20 min at 70°C under anaerobic conditions. In addition, the purified FDH displays high catalytic activity over a wide pH range. The maximal activity was observed at pH 8.5 (Fig. 2B), but the activity decreased at low pH. Although the optimum reaction pH of the enzyme was shown to be weak alkaline at pH 8.5, the FDH_{S77} had remarkable pH resistance even at significant acidic ranges below pH 4.0 (Fig. 2B). The FDH retained more than 70% of the initial activity, even after 3 h incubation at pH 3.0.

O₂-stability of the FDH_{S77}

The anaerobically purified FDH_{S77} could immediately reduce BV upon direct injection into a reaction mixture under anaerobic conditions. Surprisingly, the oxidized FDH_{S77} can be restored up to 80% of its original specific activity, even after the enzyme is exposed to air for 6 h at 4°C (Fig. 3A), but the activity was not completely recovered. The oxidized enzyme required lag times (ca. 17 min) for its full reactivation (Fig. 3B). However, the specific activity after the end of the lag time, as shown in Fig. 3B-II, was almost the same as that of the anaerobically purified enzyme of Fig. 3B-I. O₂-sensitive FDH usually shows a significant decline of specific activity, and easily loses catalytic ability after air oxidation (13,14). Compared to other bacterial FDHs, the FDH_{S77} has a remarkable O₂-stability. O₂-stable FDHs were also reported from other microorganisms, such as those of *D. desulfurican*, *D. gigas*, *D. alsaskensis*,

Rhodobacter capsulatus, and *E. coli* (15-17,25,26), however, the mechanism for how to stabilize the aerobic conditions of the O₂-stable FDHs is not yet understood.

Electrochemical experiments of the FDH_{S77}

In order to investigate how fast the purified FDH_{S77} can restore its catalytic activity from the oxidized state, the corresponding electrocatalytic activity for formate oxidation was determined by the amperometric method. Amperometric detection of the FDH_{S77} was first held under a headgas of 100% N₂ at a reference potential of -200 mV vs. Ag/AgCl electrode. The FDH_{S77} generated an extremely stable electric current of formate-oxidation at the conditions (Fig. 4). However, the current density drastically decreased toward zero when the flowing gas in the electrochemical cell was changed from N₂ to air. Surprisingly, the reduced current was recovered by continuous bubbling of N₂ to flush out the dissolved O₂ into the reaction solution. As shown in Fig. 4, reactivation of the enzyme occurred within 300 s. After substantial reduction, the current density for formate oxidation could be restored to near its initial current density after of approximately 1000 s. This intriguing result suggests that the FDH_{S77} has an excellent stability against O₂.

Kinetic properties of the FDH_{S77}

In the steady-state, kinetic parameters were determined by using the Michaelis-Menten equation at pH 7.0 and 30°C. The K_m value for formate of the FDH_{S77} was estimated to be 0.44 mM on the conditions of BV (10 mM) as an electron acceptor (Supplementary Fig. S1). This K_m value indicates that the FDH_{S77} shows substantially higher affinity for formate than other bacterial FDHs, such as the K_m values of *D. desulfurican* (21 mM) and *Clostridium pasteurium* (1.7 mM) (16,27). The limiting rate (V_{max}) was estimated as 30.4 U/mg (Supplementary Fig. S1). The turnover number (k_{cat}) was calculated as 76 s⁻¹, using the

calculated FDH_{S77} molecular mass of 150 kDa. The specificity constant (k_{cat}/K_m) for formate was $1.73 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. In the assay conditions of BV as an electron acceptor, the specific activity of FDH_{S77} reached up to 24.5 U/mg, which is almost 10-fold greater than that (2.4 U/mg) of FDH-N from *E. coli* (13).

UV-visible and ESR spectra of the purified FDH

The UV/visible spectra of air-oxidized FDH_{S77} exhibited a broad absorption peak around 400 nm and a slight shoulder in the 310 nm region (Supplementary Fig. S2), which is a typical feature of non-heme iron proteins (22,28). The purified FDH_{S77} did not contain *b*-type heme, showing no typical α -absorption peak of the reduced state around 560 nm. However, absorbance in this region around 400 nm decreased by reduction of sodium dithionite under anaerobic conditions. In the EPR studies, the oxidized FDH_{S77} was EPR silent, indicating that the active center of the enzyme was the Mo(VI) state. The formate-reduced FDH_{S77} exhibited the rhombic EPR spectrum at $g = 2.018, 1.994, \text{ and } 1.961$ at 110 K (Fig. 5). The signal is attributed to the representative signals from Mo(V) of FDH (29,30). The Mo(V) EPR signals of formate-reduced FDHs from *D. desulfuricans* were detected at g -values of 2.012, 1.996, and 1.985 and that of *Methylobacterium* sp. RXM was observed at 2.002, 1.987, and 1.959. The signal intensity increases with increasing microwave power. Although the line shape of the EPR spectra of FDH_{S77} was not completely identical to those of other FDHs, the EPR signal apparently arose from the Mo(V) state (29,30). According to the ICP-MS analysis, the purified FDH_{S77} contains molybdenum (0.97 atom/molecule), selenium (1.2 atom/molecule), and iron (17.5 atoms/molecule), but not tungsten. Based on the iron content, the FDH_{S77} may possess five [4Fe-4S] clusters; whereas FDH-N from *E. coli* contains five [4Fe-4S] clusters (18) and FDH from *D. gigas* contains four [4Fe-4S] clusters (15). The amount of iron content suggests that the purified FDH_{S77} may be slightly damaged by some degradation of iron sulfur

clusters during the procedures of solubilization and purification. On the basis of the results of the EPR spectrum and ICP-MS analysis, the purified FDH_{S77} belongs to the family of Mo containing FDH.

DISCUSSION

During the course of investigating efficient biocatalysts from microorganisms, we found an O₂-stable FDH from *Citrobacter* sp. S-77. The strain S-77 is a facultative anaerobic bacterium, which can grow well under aerobic and anaerobic growth conditions around 32 C (21). The O₂-stable FDH_{S77} may have an important role in the membrane-associated electron transport chain of *Citrobacter* sp. S-77. The O₂-stable FDH may have structural features to function efficiently under an aerobic environment. Although there is currently no defined structural information on the O₂-stable FDH_{S77}, we predict that the difference in amino acid environment in the catalytic center or electron transfer iron-sulfur clusters may be involved in the O₂-stability of the enzyme. *Citrobacter* sp. S-77 belongs to the family of *Enterobacteriaceae*, suggesting that the FDH_{S77} may have similar characteristics to those of the FDH-N from *E. coli*. The molecular weight (150 kDa) of the FDH_{S77} is significantly different to that (510 kDa) of the FDH-N from *E. coli*. The FDH-N is composed of a trimer of heterotetramers (9,13,18). However, the purified FDH_{S77} consisted of two subunits of approximately 95 kDa for the large subunit and 32 kDa for the small subunit (Fig. 1A), which is very similar to those of the periplasm-localized FDHs from *D. gigas* and *D. desulfurican* (15,16). Of all the FDHs reported so far, the heterodimeric structure of the FDH is considered to be biologically relevant. From the results of the N-terminal amino acid sequence analysis, the FDH_{S77} is highly homologous to the FDH-N from *E. coli*. In comparison to the amino acid sequences of other bacterial FDHs (www.ncbi.nlm.nih.gov), we have found that the large subunit of FDHs contains the signal sequence of a twin-arginine translocase (Tat),

350 corresponding to the initial 33 amino acid residues (9). The N-terminal sequence determined
351 for the large subunit of the purified FDH_{S77} begins after the Tat signal peptide. The
352 metalloenzyme complex of FDH is exported from cytoplasm to periplasm portion by a Tat
353 pathway (9,31), where the large subunit plays an important role in FDH secretion from
354 cytoplasm to periplasm portion across the membrane. On the other hand, the same Tat system
355 also serves as an O₂-tolerant MBH that is contained in the signal peptide sequence in the small
356 subunit, whereas the large subunit lacks the targeting signal sequence (32).
357 Membrane-anchoring proteins are commonly cleaved at the boundary surface of the inner
358 membrane or transmembrane helix when the steps of solubilization and purification are
359 performed. The exquisite example is the O₂-tolerant MBH-cytochrome *b* complex
360 (21,22,33,34). The cytochrome *b* subunit of MBH and a C-terminal transmembrane helice in
361 the small subunit were spontaneously cleaved off during solubilization and purification
362 processes. Based on the current results, we predict that the transmembrane portion of the
363 small subunit and the inner membrane protein of the cytochrome *b* subunit of the purified
364 FDH_{S77} also appear to be removed from the FDH complex during the solubilization and
365 purification steps. This might also affect the cleavage of the 95 kDa large subunit and
366 production of the 87 kDa protein fragment observed on SDS-PAGE analysis. However, the
367 cytochrome *b* subunit is an *in vivo* essential part of the FDH complex as an electron acceptor,
368 which functions as an electron transfer chain (9,18). Apart from the scientific interest in the
369 physiological role of the O₂-stable FDH along with its thermostability and pH resistance, the
370 FDH_{S77} is also of interest in the application perspective. The prominent stability of the FDH_{S77}
371 is more likely to accelerate our interest in determining further biochemical and
372 structure-functional studies that will lead to a better understanding of the molecular
373 mechanism of formate-oxidation of the O₂-stable FDH.

374

ACKNOWLEDGEMENTS

This work was supported by the World Premier International Research Center Initiative (WPI), grants-in-aid: 23655053, 25620047, 25248017, and 24109016 (Scientific Research on Innovative Areas “Stimuli-responsive Chemical Species”) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and the Basic Research Programs CREST Type, "Development of the Foundation for Nano-Interface Technology" from JST, Japan.

References

- Berg, I. A.:** Ecological aspects of the distribution of different autotrophic CO₂ fixation pathways, *Appl. Environ. Microbiol.*, **77**, 1925-1936 (2011).
- Appel, A. M., Bercaw, J. E., Borcarsly, A. B., Dobbek, H., DuBois, D. L., Dupuis, M., Ferry, J. G., Fujita, E., Hille, R., Kenis, P. J. A., and other 10 authors:** Frontiers, opportunities, and challenges in biochemical and chemical catalysis of CO₂ fixation, *Chem. Rev.*, **113**, 6621-6658 (2013).
- Ihara, M., Kawano, Y., Urano, M., and Okabe, A.:** Light driven CO₂ fixation by using cyanobacterial photosystem I and NADPH-dependent formate dehydrogenase, *PLoS One.*, **8**, e71581 (2013).
- Ferry, J. G.:** Formate dehydrogenase, *FEMS Microbiol. Rev.*, **87**, 377-382 (1990).
- Gonzalez, P. J., Rivas, M. G., Mota, C. S., Brondino, D. C., Moura, I., and Moura, J. J. G.:** Periplasmic nitrate reductases and formate dehydrogenases: biological control of the chemical properties of Mo and W for fine tuning of reactivity, substrate specificity and metabolic role, *Coord. Chem. Rev.*, **257**, 315-331 (2013).
- Tishkov, V. I. and Popov, V. O.:** Protein engineering of formate dehydrogenase, *Biomol. Eng.*, **23**, 89-110 (2006).

400 + " **Yoshimoto, M., Kunihiro, N., Tsubomura, N., and Nakayama, M.:** Preparation of
 401 liposome-coupled NADH and evaluation of its affinity toward formate dehydrogenase
 402 based on deactivation kinetics of the enzyme, *Colloids Surf., B*, **109**, 40-44 (2013).

403 , " **Joó, F.:** Breakthroughs in hydrogen storage—formic acid as a sustainable storage material
 404 for hydrogen, *ChemSusChem*, **1**, 805-808 (2008).

405 - " **Jormakka, M., Byrne, B., and Iwata, S.:** Formate dehydrogenase – a versatile enzyme
 406 in changing environments, *Curr. Opin. Struct. Biol.*, **13**, 418-423 (2003).

407 %\$ " **Schuchmann, K. and Müller, V.:** Direct and reversible hydrogenation of CO₂ to
 408 formate by a bacterial carbon dioxide reductase, *Science*, **342**, 1382-1385 (2013).

409 %% " **Yadav, R. K., Baeg, J.-O., Oh, G. H., Park, N.-J., Kong, K.-J., Kim, J., Hwang, D.**
 410 **W., and Biswas, S. K.:** A photocatalyst-enzyme coupled artificial photosynthesis system
 411 for solar energy in production of formic acid from CO₂, *J. Am. Chem. Soc.*, **134**,
 412 11455-11461 (2012).

413 %& " **Reda, T., Plugge, C. M., Abram, N. J., and Hirst, J.:** Reversible interconversion of
 414 carbon dioxide and formate by an electroactive enzyme, *Proc. Natl. Acad. Sci. USA*, **105**,
 415 10654-10658 (2008).

416 % " **Enroch, H. G. and Lester, R. L.:** The purification and properties of formate
 417 dehydrogenase and nitrate reductase from *Escherichia coli*, *J. Biol. Chem.*, **250**,
 418 6693-6705 (1975).

419 % " **Axley, M. J., Grahame, D. A., and Stadtman, T. C.:** *Escherichia coli*
 420 formate-hydrogen lyase: purification and properties of the selenium-dependent formate
 421 dehydrogenase component, *J. Biol. Chem.*, **265**, 18213-18218 (1990).

422 % " **Raaijmakers, H., Teixeira, S., Dias, J. M., Almendra, M. J., Brondino, C. D.,**
 423 **Moura, I., Moura, J. J., and Romão, M. J.:** Tungsten-containing formate

xdehydrogenase from *Desulfovibrio gigas*: metal identification and preliminary structural data by multi-wavelength crystallography, *J. Biol. Inorg. Chem.*, **6**, 398-404 (2001).

%" **Costa, C., Teixeira, M., LeGall, J., Moura, J. J. G., and Moura, I.:** Formate dehydrogenase from *Desulfovibrio desulfuricans* ATCC 27774: isolation and spectroscopic characterization of the active sites (heme, iron-sulfur centers and molybdenum), *J. Biol. Inorg. Chem.*, **2**, 198-208 (1997).

%" **Brondino, C. D., Passeggi, M. C. G., Caldeira, J., Almendra, M. J., Feio, M. J., Moura, J. J. G., and Moura, I.:** Incorporation of either molybdenum or tungsten into formate dehydrogenase from *Desulfovibrio alaskensis* NCIMB 13491; EPR assignment of the proximal iron-sulfur cluster to the pterin cofactor in formate dehydrogenases from sulfate-reducing bacteria, *J. Biol. Inorg. Chem.*, **9**, 145-151 (2004).

%" **Jormakka, M., Törnroth, S., Byrne, B., and Iwata, S.:** Molecular basis of proton motive force generation: structure of formate dehydrogenase-N, *Science*, **295**, 1863-1868, (2002).

%" **Kim, S., Seol, E., Raj, S. M., Park, S., Oh, Y.-K., and Ryu, D. D. Y.:** Various hydrogenases and formate-dependent hydrogen production in *Citrobacter amalonaticus* Y19, *Int. J. Hydrogen Energy*, **33**, 1509-1515 (2008).

88" **Zatsepin, S. S. and Netrusov, A. I.:** Enzyme activity of the formate hydrogenlyase complex in *Citrobacter freundii*, *Mikrobiologiya*, **53**, 246-250 (1984).

88" **Eguchi, S., Yoon, K.-S., and Ogo, S.:** O₂-stable membrane-bound [NiFe]hydrogenase from a newly isolated *Citrobacter* sp. S-77, *J. Biosci. Bioeng.*, **114**, 479-484 (2012).

88" **Yoon, K.-S., Fukuda, K., Fujisawa, K., and Nishihara, H.:** Purification and characterization of a highly thermostable, oxygen-resistant, respiratory [NiFe]-hydrogenase from a marine, aerobic hydrogen-oxidizing bacterium *Hydrogenovibrio marinus*, *Int. J. Hydrogen Energy*, **36**, 7081-7088 (2011).

- 449 23. **Matsudaira, P.:** Sequence from picomole quantities of proteins electroblotted onto
450 polyvinylidene difluoride membranes, *J. Biol. Chem.*, **262**, 10035-10038 (1987).
- 451 24. **Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.:** Protein
452 measurement with the folin phenol reagent, *J. Biol. Chem.*, **193**, 265-275 (1951).
- 453 25. **Hartmann, T. and Leimkühler, S.:** The oxygen-tolerant and NAD⁺-dependent formate
454 dehydrogenase from *Rhodobacter capsulatus* is able to catalyze the reduction of CO₂ to
455 formate, *FEBS J.*, **280**, 6083-6096 (2013).
- 456 26. **Sousa, P. M., Videira, M. A., and Melo, A. M.:** The formate: oxygen oxidoreductase
457 supercomplex of *Escherichia coli* aerobic respiratory chain, *FEBS Lett.*, **587**, 2559-2564
458 (2013).
- 459 27. **Liu, C.-L. and Mortenson, L. E.:** Formate dehydrogenase of *Clostridium pasteurianum*,
460 *J. Bacteriol.*, **159**, 375-380 (1984).
- 461 28. **Mota, C. S., Valette, O., González, P. J., Brondino, C. D., Moura, J. J. G., Moura, I.,
462 Dolla, A., and Rivas, M. G.:** Effects of molybdate and tungstate on expression levels
463 and biochemical characteristics of formate dehydrogenases produced by *Desulfovibrio*
464 *alaskensis* NCIMB 13491, *J. Bacteriol.*, **193**, 2917-2923 (2011).
- 465 29. **Rivas, M. G., González, P. J., Brondino, C. D., Moura, J. J. G., and Moura, I.:** EPR
466 characterization of the molybdenum(V) forms of formate dehydrogenase from
467 *Desulfovibrio desulfuricans* ATCC 27774 upon formate reduction, *J. Inorg. Biochem.*,
468 **101**, 1617-1622 (2007).
- 469 30. **Duarte, R. O., Reis, A. R., Gírio, F., Moura, I., Moura, J. J. G., and Collaço, T. A.:**
470 The formate dehydrogenase isolated from the aerobe *Methylobacterium* sp. RXM is a
471 molybdenum-containing protein, *Biochem. Biophys. Res. Commun.*, **230**, 30-34 (1997).
- 472 31. **Sargent, F., Berks, B. C., and Palmer, T.:** Assembly of membrane-bound respiratory
473 complexes by the Tat protein-transport system, *Arch. Microbiol.*, **178**, 77-84 (2002).

32. Wu, L.-F., Chanal, A., and Rodrigue, A.: Membrane targeting and translocation of bacterial hydrogenases, Arch. Microbiol. **173**, 319-324 (2000).
33. Fritsch, J., Lenz, O., and Friedrich, B.: The maturation factors HoxR and HoxT contribute to oxygen tolerance of membrane-bound [NiFe] hydrogenase in *Ralstonia eutropha* H16, J. Bacteriol., **193**, 2487-2497 (2011).
34. Shomura, Y., Yoon, K.-S., Nishihara, H., and Higuchi, Y.: Structural basis for a [4Fe-3S] cluster in the oxygen-tolerant membrane-bound [NiFe]-hydrogenase, Nature, **479**, 253-256 (2011).

Figure legends

Fig. 1. (A) SDS-PAGE (12.5%) gel of the purified FDH_{S77}. Lane 1, low-molecular weight standard maker proteins (M_r s, 14,400 to 97,000); lane 2, 5 μ g of the purified FDH_{S77}. (B) Comparison of N-terminal amino acid sequences of FDHs. The sequences of the FDH_{S77} were determined in this study. The sequences from other FDHs were extracted from the protein database of NCBI (www.ncbi.nlm.nih.gov). (C) Native-PAGE (5-20% gradient) analysis of the purified FDH_{S77}. Lane 1, protein stain of 10 μ g of the purified FDH_{S77}, lane 2: activity stain of 10 μ g of the purified FDH_{S77}. (D) The gel filtration behavior of Superdex 200 column of the FDH_{S77} as the final purification step. (E) Calibration curve for the determination of molecular weight of the purified FDH_{S77} by gel filtration using a Superose 12 HR (10/300; GE Healthcare, UK) column. The FDH_{S77} eluted at a position corresponding to 150 kDa. The column was calibrated with standard molecular weight markers (Bio-Rad Laboratories Inc.): bovine thyroglobulin (M_r = 670,000), bovine γ -globulin (M_r = 158,000), chicken ovalbumin (M_r = 44,000), horse myoglobin (M_r = 17,000), and vitamin B₁₂ (M_r = 1,350). In all cases, 20

499 mM Tris-HCl buffer, pH 8.0 containing 0.2 M NaCl and 0.2 % SB3-12 was used as the eluted
500 buffer.

501
502 **Fig. 2.** Influences of pH value and temperature for the activity of the purified FDH_{S77}. (A)
503 Influences of temperature: thermostability of the purified FDH_{S77} after treatment for 20 min
504 between 0 and 80°C under N₂ (closed circles) and optimal reaction temperature of the FDH_{S77}
505 (open squares). The FDH activity was measured at pH 7.0. (B) Effect of pH: pH stability of
506 the purified FDH_{S77} after treatment for 3 h at 4°C between pH 3.0 and 10.0 under N₂ (open
507 squares) and the optimum reaction pH of the purified FDH_{S77} (closed circles). The obtained
508 data were calculated by the means of at least three independent experiments.

509
510 **Fig. 3.** O₂ stability of the purified FDH_{S77}. (A) Long-term O₂-stability of the purified FDH_{S77}.
511 The purified FDH_{S77} was exposure to air, then incubated under air at 4°C. The remaining
512 activities periodically were measured during the air oxidation procedure. To remove dissolved
513 O₂ from the enzyme solution, the samples were flushed with N₂ for 5 min. All enzyme assays
514 were initiated by the direct injection of prepared enzyme solution into the reaction mixture
515 with a syringe. (B) Required incubation time to recover activity of the enzyme: the
516 anaerobically purified FDH (A, solid line); the air-oxidized FDH for 6 h at 4°C (B, dotted
517 line). All assays were performed under the same buffer conditions of 50 mM MOPS buffer at
518 pH 7.0 and at 30°C. The obtained data were calculated by the means of at least three
519 independent experiments.

520
521 **Fig. 4.** Amperometric *i-t* curve of the purified FDH_{S77}. The catalytic current of
522 formate-oxidation of FDH_{S77} under 100% N₂ at 30°C was detected for 1000 s. After recording

the current density, the bubbling gas was changed from N₂ to dry air for 180 s. The dissolved O₂ into the solution was then removed by flushing with N₂ gas. Experimental data was recorded under the conditions with a potential of -200 mV vs. Ag/AgCl electrode with rotating the working electrode at 3,500 rpm at 30°C using a gas flow rate of 1000 scc/min.

Fig. 5. EPR spectra of formate-reduced FDH_{S77}. The EPR experiments were performed on the conditions of freshly prepared enzyme (10 mg/mL) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 0.2% SB3-12. The reduced FDH_{S77} was prepared by addition of 10 mM formate into the enzyme solution and the sample was then incubated for 20 min at 30°C under anaerobic conditions. Experimental conditions: 1 mW (dotted line) and 10 mW (solid line) microwave power; 80 mT modulation amplitude; 9.20 GHz microwave frequency.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5