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

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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 O_2-stability along with thermostability and pH resistance. This is the first report of the purification and characterization of a FDH from *Citrobacter* species.

[**Key words:** Molybdenum-containing formate dehydrogenase; *Citrobacter* sp. S-77; Formate oxidation; O_2-stability; Thermostability; pH resistance]

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 CO_2 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, and so on (1–3). Among them, FDH is one of the most attractive metalloenzymes that catalyzes the reversible oxidation of formate to produce two-electrons and CO_2, 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 homogeneous, formate-oxidizing biocatalyst in safe fuel cell systems. Additionally, FDH has a great potential as an effective biocatalyst for conversion of CO_2 to make formate as an alternative fuel (7–12). Compared to H_2, formate is a favorable 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 CO_2 to formate (10–12). However, the enzyme is very sensitive to O_2, easily losing its catalytic activity after air oxidation (13,14). The O_2-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 O_2-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 *Citrobacter amalonaticus* (19) and *Citrobacter 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

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(21), which has remarkable O_2-stability and high H_2-oxidation. Like the MBH from *Citrobacter* sp. S-77, the purified FDH_{S77} displays a remarkable O_2-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 CO_2 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 $MgSO_4 \cdot 7H_2O$, 3.0 g $(NH_4)_2SO_4$, 2.0 g K_2HPO_4, 1.0 g KH_2PO_4, 1.0 g D-glucose, 2.0 g sodium formate, 0.2 g ammonium iron(III) citrate, and 0.1 g of $CaCl_2$ and the minor mineral components of 0.1 μM Na_2WO_4, 0.1 μM Na_2MoO_4, and 0.2 μM $Na_2Se_2O_3$. The strain S-77 easily entered the stationary phase after 2 days of culture at 32°C. The cells were harvested by centrifugation at 9000 ×*g* for 20 min. The precipitated pellet was frozen in liquid N_2 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 3000 ×*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% N_2 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% N_2 and 2% H_2. All buffers were repeatedly degassed and flushed with N_2 and were stored in the anaerobic chamber. Protein purification was performed in the Coy anaerobic chamber using an AKTA-FPLC system (GE Healthcare, UK). 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 and 100 mM KP buffer. The active pools of FDH were directly applied onto a Q Sepharose HP (1.6 × 12 cm; GE Healthcare) 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). 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 0.25–0.4 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, USA) and then loaded onto a Superdex 200 column (1.6 × 50 cm; GE Healthcare) pre-equilibrated with 20 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 nondenaturating 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 N_2 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 N_2 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 ($\varepsilon_{600\ nm} = 8.3\ mM^{-1}\ cm^{-1}$) (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, USA). 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 O_2-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 O_2 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 N_2 for 5 min to remove the dissolved trace O_2 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 N_2. 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 N_2 at each measuring temperature. For the thermostability of the purified FDH_{S77}, the enzyme solution (0.1 mg/mL) in a 20 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 N_2. 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 O_2-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 N_2 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 N_2 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