Supporting Information

Multiple Electron Transfer Pathways of Tungsten-containing Formate Dehydrogenase in Direct Electron Transfer-type Bioelectrocatalysis

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Experimental

Reagent and chemicals

4-Mercaptopyridine (4-MP) was purchased from Tokyo Chemical Industry (Tokyo, Japan). NADH was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All the solutions were prepared using distilled water. FoDH1 was purified according to a literature procedure¹.

Electrode fabrication

4-Aminothiophenol (4-ATP) and 4-mercaptopyridine (4-MP)-modified gold porous electrodes were prepared according to the literature². Briefly, gold electrodes (3 mm in diameter, BAS, USA) were polished with an alumina slurry (1 μ m and 0.05 μ m successively) and sonicated with distilled water. The gold electrodes were anodized at 1.19 V for 120 s to fabricate a porous gold electrode (PGE). To modify 4-ATP or 4-MP on PGEs, the electrode was immersed in ethanol solutions containing 10 mM 4-ATP or 4-MP. Then, these electrodes were kept at 4 °C overnight. These electrodes are referred to as 4-ATP/PGE and 4-MP/PGE.

Electrochemical measurements

Electrochemical measurements were performed using an ALS 701E electrochemical analyzer (BAS, USA). Ag|AgCl(sat.KCl) and a platinum wire, were used as the reference and counter electrodes, respectively. All potentials in this study are referred to the reference electrode. All measurements were carried out in potassium phosphate buffer (pH 7.0) under quiescent and Ar-saturated conditions (25 ± 2 °C).

FoDH1 assays

FoDH1 activity assays were performed in 1-cm light-path cuvettes, when the substrates were 5 mM potassium formate and 5 mM NAD⁺. 0.1-cm light-path cuvettes were used when the substrates were saturated CO₂ and 1.5 mM NADH. Enzyme reactions were initiated by adding the FoDH1 solution, and the change in absorbance at 340 nm due to the production or consumption of NADH was measured using a Shimadzu UV 1900-I system. One unit of FoDH1 activity was defined as the amount of FoDH1 that catalyzes the reduction of 1 µmol of NAD⁺ or the oxidation of 1 µmol of NADH per minute at pH 7.0. The extinction coefficient for NADH at 340 nm was set to 6.3×10^3 cm ⁻¹ M⁻¹. The protein concentration was estimated using a DC protein assay kit (Bio-Rad,

USA) with bovine serum albumin as the standard.

Cryo-EM data collection

A 2.7 μ L protein solution of the FoDH1 complex (5 mg mL⁻¹) was applied to Quantifoil Cu R1.2/1.3 holey carbon grids and frozen in liquid ethane using a Vitrobot IV system (FEI, 4 °C and 100 % humidity, 3 s blotting time, and 2 s drain time). Sample data were collected on a CRYO ARM 300 (JEOL, Japan) system equipped with a cold field-emission electron gun operated at 300 kV, an Ω -type energy filter with a 20-eV slit width, and a K3 direct electron detector camera (Gatan, USA). Serial-EM³, an automated data acquisition program, which was used to collect cryo-EM image data. Movie frames were recorded using the K3 camera at a calibrated magnification of ×60,000 corresponding to a pixel size of 0.87 Å with a setting defocus range from -1.0 to -2.0 μ m. The data were collected with total exposure of 3 s fractionated into 40 frames, with a total dose of ~80 electrons Å⁻² in the counting mode. A total number of 7,650 movies were collected.

Molecular modeling

Atomic model building of FoDH1 was carried out using COOT (Crystallographic Object-Oriented Toolkit). PHENIX was used for real-space refinement of the model based on the map of FoDH1 obtained by cryo-EM. Cofactors were added on the basis of the information described in the literature⁴. Our atomic model covers residues 69–981 and 6–565 in FoDH1A (990 residues) and FoDH1B (572 residues), respectively.

SDS-PAGE of FoDH1

The purified FoDH1 solution⁵ was separated on a 10% polyacrylamide gel and stained with Coomassie brilliant blue R250 (Fig. S1).



Figure S1. SDS-PAGE analysis result of purified FoDH1. Lane (A): standard proteins, β -galactosidase (111 kDa), bovine serum albumin (85.9 kDa), ovalbumin (46.7 kDa), and carbonic anhydrase (36.2 kDa). Lane (B): 5 µg of purified FoDH1 solution. Two major bands corresponding to α -subunit (110 kDa) and β -subunit (60 kDa) of FoDH1 were identified.

Structural analysis

Image processing and 3D reconstruction

Motion correction was carried out by RELION 3.1⁶ to align all FoDH1 complex micrographs, and the CTF parameters were estimated using CTFFIND4⁷. The FoDH1 complexes were automatically selected via auto-picking by using a boxnet in Warp⁸, and the selected 1,594,212 particles were extracted into a box of 256 × 256 pixels (Figs. S2 a,b). For the FoDH1 complex, 807,411 particles were selected after 2D classification using RELION 3.1. The initial 3D reference was calculated from the 3D initial mode of RELION 3.1. The first 3D classification into three classes with C1 symmetry resulted in one class (427,802 particles). These processes were performed by binning 2. 3D refinement was performed and employed as an additional CTF refinement procedure and for particle polishing. The map at 2.5 Å resolution after solvent mask post-processing was obtained. The final map at 2.2 Å resolution after solvent mask post-processing was obtained after further CTF refinement and particle polishing (Figs. S2c–e; EMDB ID: 32151, PDB ID: 7VW6).

Data and code availability

The cryo-EM density map of FoDH1 was deposited into EMDataBank with the accession code EMD-32151. The atomic coordinates of the FoDH1 complex have been deposited in Protein Data Bank with the accession code PDB ID: 7VW6.



Figure S2. Cryo-EM analysis result of FoDH1. (a) A representative cryo-EM picture of FoDH1 data collection. (b) 2D class averages of various views of FoDH1. (c) The work process for cryo-EM single-particle structural analysis of FoDH1. (d) The local resolution of FoDH1 density map colored from red (2.60 Å) to blue (1.96 Å). (e) FSC of the final density map of FoDH1 reconstructed with C1 symmetry.

FoDHI
(EMDB-32151)
(PDB 7VW6)
<pre><pre></pre></pre>
60,000
300
80
-1.02.0
0.87
C1
1,594,212
427,802
2.2
0.143
1.96-2.60
de novo
21
0.143
80-2.1
-40.2
10.2
11485
1473
11
24.63
17.25
0.25
0.52
1.48
2.83
2.03
97.00
3.00
0

Table S1. EM data collection, processing, and refinement statistics



Figure S3. Structural model of the W-pterin active site in FoDH1A. The grey mesh represents the electron density map. Tungsten was coordinated by the atom and the residues are shown in red. This model was constructed using PyMOL (The PyMOL molecular Graphics System, Version2.0 Schrödinger, LLC).

Table S2. Clash scores, B-factors, and distances between W and each ligand. Clash scores and B-factors were calculated by PHENIX. B-factor on this table refers to the mean of Ligand B-factor. Distances were calculated using PyMOL. The chemical species for sixth ligand were selected from the previous study^{4,9,10}

Sixth ligand	Clash score	B-factor / Å ²	Distance / Å
UNX	2.83	17.25	2.5
Ο	2.91	16.66	2.2
S	3.00	16.42	2.2
H_2S	3.27	17.36	1.8 (for S atom) 2.6 (for H atom)



Figure S4. Distribution of edge-to-edge distances from each [Fe-S] cluster to α carbon of amino acid residues located on the enzyme surface. The amino acid residues determined to be solvent-accessible from PISA web service were considered to be located on the enzyme surface.



Figure S5. Spherical representation of amino acid residues in close proximity to each [Fe-S] clusters. Amino acid residues are colored based on their distance between α -carbon and the corresponding [Fe-S] cluster (A1~A4, B1, and B2) (orange: 6~8 Å, cyan: 8~11 Å, and pink: 11~14 Å)



Figure S6. Surface charges of the FoDH1 calculated by PDB2PQRS in pH 7 (blue: positive charge, red: negative charge). The top one is identical to Fig. 2(a) in the text, and the bottom one is rotated 180 degrees horizontally.

Kinetic analysis of DET-type bioelectrocatalysis of FoDH1

Under the conditions where the concentration of substrate is sufficient to avoid the limitation of mass transfer, the observed steady-state current density (j) at 4-ATP/PGE and 4-MP/PGE can be given by

$$j = j_{electro-enz} \tag{1}$$

where $j_{electro-enz}$ is the steady-state catalytic current density determined by DET-type bioelectrocatalysis, and the limiting value of $j_{electro-enz}$ is referred to as j_{cat} and is defined as follows:

$$j_{cat} = n_{sub} F k_c \Gamma_{E,eff} \tag{2}$$

where *n*, *F*, k_c , and $\Gamma_{E,eff}$ are the number of electrons in the catalytic reaction of the substrate (= 2), Faraday constant, catalytic constant, and surface concentration of the effective modified enzyme on the electrode, respectively. We attempted to estimate the thermodynamic and kinetic parameters of DET-type bioelectrocatalysis at the FoDH1-adsorbed electrodes based on a random orientation model^{11,12}. In this model, $j_{electro-enz}$ is expressed as follows^{12,13}:

$$j_{electro-enz} = \frac{j_{cat}}{\beta \Delta d(1+\eta)} ln \left| \frac{\frac{k^{\circ}_{max}}{k_c}(1+\eta) + \eta^{\alpha}}{\frac{k^{\circ}_{max}}{k_c}(1+\eta) \exp(-\beta \Delta d) + \eta^{\alpha}} \right|$$
(3)

where k_{max}° , β , Δd , and α are, respectively, the standard rate constant of the heterogeneous ET at the closest approach in the best orientation of the enzyme, the decay coefficient of the long-range ET (assumed to be 1.4 Å^{-1} for proteins), the difference in the distance between the closest and farthest approaches of the redox center of the electroactive enzyme, and the transfer coefficient (= 0.5, in general)¹⁴. η is defined as follows:

$$\eta = \exp\left\{\frac{n_E F}{RT} \left(E^{\circ}_E - E\right)\right\}$$
(4)

where n_E , E, $E_E^{o'}$, R, and T are the number of electrons in the rate-determining step of the heterogeneous ET (=1 in general), the electrode potential, the formal potential of the electrode-active site, the gas constant, and the absolute temperature, respectively. The background currents were subtracted from the total currents, and corrected current densities were used for the analysis. Equation (3) was fitted to the steady-state catalytic waves (NADH oxidation at 4-ATP/PGE and 4-MP/PGE, as well as the HCOO⁻ oxidation

at 4-MP/PGE) using non-linear regression analysis by Gnuplot®, using $E_E^{\circ} \Delta d$, k_{max}°/k_c , and j_{cat} as adjustable parameters. For the kinetic analysis, we used the data for a forward scan in each CV. A good fit can be obtained in the analysis, as described by the solid lines in Fig. S6. The refined parameters are listed in Table S3.



Figure S7. Background-subtracted catalytic voltammograms of (a) NADH oxidation at 4-ATP/PGE, (b) NADH oxidation at 4-MP/PGE, and (c) HCOO⁻ oxidation at 4-MP/PGE. The circles indicate the experimental data used for this kinetic analysis. The dashed lines indicate the refined curves obtained by non-linear regression analysis based on Eq. (3).

Table S3. Refined parameters obtained by the non-linear regression analysis of catalytic voltammograms. The errors were evaluated from Student's *t*-distribution at a 90 % confidence level. The number of each sample is 4.

	$E_E^{\circ'} / \mathbf{V}$	k_{max}°/k_c	Δd / Å	j_{cat} / $\mu A \text{ cm}^{-2}$
NADH oxidation at 4-ATP/PGE	-0.48 ± 0.01	$(2\pm3)\times10^2$	9 ± 1	70 ± 30
NADH oxidation at 4-MP/PGE	-0.46 ± 0.02	5 ± 7	6 ± 1	30 ± 20
HCOO ⁻ oxidation at 4-MP/PGE	-0.41 ± 0.02	$(4\pm4)\times10^3$	9.5 ± 0.8	20 ± 10

Specific activities of FoDH1

The activities of FoDH1 were determined as described in the Experimental section. The results obtained by spectrophotometry are shown in Figure S8, and the specific activities are listed in Table S4. When the substrates were CO_2 and NADH, the change in absorbance in the first 15 s was used for the activity calculation. Specific activities were calculated from the results of the activity assay and protein quantification.



Figure S8. Change in the absorbance at 340 nm detected by spectrophotometry in solutions containing (a) saturated CO₂, 1.5 mM NADH, and 73 μ g mL⁻¹ FoDH1 solution and (b) 5 mM HCOO⁻, 5 mM NAD⁺ and 0.3 μ g mL⁻¹ FoDH1 solution (solid lines). Solid lines indicate the three independent experiments. Dashed lines indicate the change in the absorbance at 340 nm without FoDH1.

Table S4. Specific activities obtained from spectrophotometric measurements. In calculating the error values, the Student's *t*-distribution was assumed and the confidence level was set at 90 %. The number of repeats for each sample is 3.

Substrates	Specific activity (U mg ⁻¹)
CO ₂ and NADH	3.6 ± 0.7
HCOO ⁻ and NAD ⁺	99 ± 9

Molecular phylogenetic analysis of formate dehydrogenase variants

The sequences of various formate dehydrogenases were selected according to the literature³. We used the neighbor-joining method to determine evolutionary relationships in the form of a phylogenetic tree¹⁵. Phylogenetic analysis was conducted using the local alignment search tool (NCBI) Multiple Alignment (COBALT)¹⁶. The number of iron-sulfur clusters in the formate dehydrogenases is listed in Table S5.



Figure S9. Phylogenetic affiliation of formate dehydrogenase variants. PDB accession codes are added for those with 3-D structures clarified. The scale bar represents the number of amino acid substitutions per sequence¹⁷. (Abbreviations: Cg = Corynrbacterium glutamicum; Ec = Escherichia coli; Ca = Clostridium acidurici; Cp = Clostridium pasteurianum; Me = Methylorubrum extorquens; Ct = Clostridium thermoaceticum; Rc = Rhodobacter capsulatus; Re = Ralstonia eutropha; Mt = Methylosinus trichosporium; Rp = Rhodopseudomonas palustris)

	4Fe-4S	2Fe-2S
CgFoDH	1	0
<i>Ес</i> FoDH-Н	1	0
CaFoDH2	4	1
<i>Cp</i> FoDH	5	0
<i>Me</i> FoDH1 (this study)	4	2
<i>Ct</i> FoDH	5	0
<i>Rc</i> FoDH	5	2
<i>Re</i> FoDH	5	2
<i>Mt</i> FoDH	5	2
<i>Rp</i> FoDH	5	2

Table S5. Number of iron-sulfur clusters in various formate dehydrogenases.

References

- K. Sakai, B. Hsieh, A. Maruyama, Y. Kitazumi, O. Shirai and K. Kano, *Sensing and Bio-Sensing Research*, 2015, 5, 90–96.
- 2 M. Miyata, K. Kano, O. Shirai and Y. Kitazumi, *Electrochemistry*, 2020, 88, 444–446.
- 3 D. N. Mastronarde, *Journal of Structural Biology*, 2005, **152**, 36–51.
- 4 T. Hartmann, N. Schwanhold and S. Leimkühler, *Biochimica et Biophysica Acta (BBA) -Proteins and Proteomics*, 2015, **1854**, 1090–1100.
- 5 M. Laukel, L. Chistoserdova, M. E. Lidstrom and J. A. Vorholt, *European Journal of Biochemistry*, 2003, **270**, 325–333.
- 6 J. Zivanov, T. Nakane, B. O. Forsberg, D. Kimanius, W. J. H. Hagen, E. Lindahl and S. H. W. Scheres, *eLife*, 2018, 7, 1–22.
- 7 A. Rohou and N. Grigorieff, *Journal of Structural Biology*, 2015, **192**, 216–221.
- 8 D. Tegunov and P. Cramer, *Nature Methods*, 2019, **16**, 1146–1152.
- C. Radon, G. Mittelstädt, B. R. Duffus, J. Bürger, T. Hartmann, T. Mielke, C. Teutloff, S. Leimkühler and P. Wendler, *Nature Communications*, 2020, 11, 1912.
- 10 B. R. Duffus, P. Schrapers, N. Schuth, S. Mebs, H. Dau, S. Leimkühler and M. Haumann, *Inorganic Chemistry*, 2020, 59, 214–225.
- C. Léger, A. K. Jones, S. P. J. Albracht and F. A. Armstrong, *The Journal of Physical Chemistry B*, 2002, 106, 13058–13063.
- 12 T. Adachi, K. Sowa, Y. Kitazumi, O. Shirai and K. Kano, *Bioelectrochemistry*, 2022, 143, 107992.
- H. Xia, Y. Kitazumi, O. Shirai and K. Kano, *Journal of Electroanalytical Chemistry*, 2016, 763, 104–109.
- C. C. Moser, J. M. Keske, K. Warncke, R. S. Farid and P. L. Dutton, *Nature*, 1992, 355, 796–802.
- 15 N. Saitou and M. Nei, *Molecular Biology and Evolution*, 1987, 4, 406–425.
- 16 J. S. Papadopoulos and R. Agarwala, *Bioinformatics*, 2007, 23, 1073–1079.
- 17 NickV. Grishin, Journal of Molecular Evolution, 1995, 41, 675–679.