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Kyoto University
Bioelectrochemical Characterization of Tungsten-Containing Formate Dehydrogenase and Development of Bioelectrocatalytic Interconversion System between Carbon Dioxide and Formate

Kento Sakai

2018
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General Introduction

In recent years, the technology of capturing and storing renewable energy has been extensively discussed and investigated. The reduction of carbon dioxide (CO₂) to generate reduced carbon compounds for use as fuels and chemical feedstocks is an essential requirement for carbon based sustainable energy economy. An interconversion system between formic acid and CO₂ is one of essential requirements for the sustainable economy. Formic acid is the first stable intermediate during the reduction of CO₂ to methanol or methane. It is increasingly recognized as not only a new energy source for electric generation but also as an energy and carbon source for microorganisms to produce high-molecular-weight hydrocarbons. In addition, it can be easily be handled, stored, and transported as formate (HCOO⁻). However, when CO₂ is reduced and HCOO⁻ is oxidized directly on electrodes, carbon monoxide is generated and quite high overpotential is required. One of the most promising strategies for solving these issues is the utilization of enzymes as catalysts. Enzymes allow the system to function in a specific biological reaction under mild conditions. The electro-enzymatic devices can be used as energy conversion system such as HCOO⁻/oxygen (O₂) biofuel cells and an efficient bioelectrochemical system of the CO₂ reduction.

In this research, the author aimed to construct a bioelectrocatalytic interconversion system between CO₂ and HCOO⁻. The author focused on the catalytic properties of tungsten-containing formate dehydrogenase (FoDH1; EC 1.2.1.2) from Methylobacterium extorquens AM1 to the interconversion system. To utilize FoDH1 as an electrocatalyst, it is necessary to construct a bioelectrocatalytic system, in which an enzyme reaction and an electrode reaction are coupled. The reaction is classified into two types: mediated electron transfer (MET) and direct electron transfer (DET). In the MET-type system, an artificial redox mediator shuttles electrons between an electrode and an enzyme. In the DET-type system, an enzyme itself directly communicate with an electrode.

In chapter 1, the author focused on the bioelectrocatalysis with FoDH1. In the DET-type bioelectrocatalysis of FoDH1, it is very important to utilize mesoporous carbon electrodes because the interfacial electron transfer rate constant of FoDH1 is not so fast. Since the rate constant increases exponentially upon decreasing the distance between the electrode surface and the redox site of the enzyme, the author developed some methods to achieve favorite orientation of FoDH1 onto the electrode surface. In addition, the author investigated the electrochemical characteristics of FoDH1 with bioelectrochemistry. In the MET-type bioelectrocatalysis of FoDH1, the author has evaluated the bi-molecular reaction rate constants between FoDH1 and mediators and NAD⁺. They show the property called a linear free energy relationship (LFER), indicating that FoDH1 would have no specificity to NAD⁺. Similar LFER is also observed for the catalytic reduction of CO₂. The reversible reaction between HCOO⁻ and CO₂ through FoDH1 has been realized by using methyl viologen (MV).
as a mediator and by adjusting pH from the thermodynamic viewpoint.

In chapter 2, the author has constructed two novel electro-enzymatic devices by utilizing the bioelectrocatalysis with FoDH1. One of them is a high power HCOO⁻/CO₂ biofuel cell. The bioanode comprises FoDH1 and a viologen-functionalized polymer, whereas the biocathode comprises bilirubin oxidase and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate). The biofuel cell shows the highest power density reported for the biofuel cells to date. The other is an efficient bioelectrocatalytic CO₂ reduction system using gaseous CO₂ directly. In this work, FoDH1 was used as a catalyst and was absorbed on a Ketjen Black (KB)-modified electrode. We used 1,1'-trimethylene-2,2'-bipyridinium dibromide as a mediator, and the hydrophobicity of the FoDH1-absorbed electrode was optimized according to the weight ratio of the polytetrafluoroethylene binder to KB.
Chapter 1

Bioelectrochemical Characterization of Tungsten-Containing Formate Dehydrogenase from *Methylobacterium extorquens* AM1

1-1. Direct electron transfer-type bioelectrocatalytic interconversion of carbon dioxide/formate and NAD\(^+/\)NADH redox couple with tungsten-containing formate dehydrogenase

Abstract

Tungsten-containing formate dehydrogenase (FoDH1) with a molecular mass of 170 kDa from *Methylobacterium extorquens* AM1 catalyzes the oxidation of formate (HCOO\(^-\)) to carbon dioxide (CO\(_2\)) with NAD\(^+\) as a natural electron acceptor in solution. FoDH1 does not produce any direct electron transfer (DET)-type bioelectrocatalytic wave at planar electrodes, but can adsorb on and communicate with mesoporous carbon electrodes. The curvature effect of mesoporous structures seems to increase the number of enzymes with orientations suitable for electrochemical communication. However, adsorption proceeds slowly on Ketjen Black-modified electrode and the catalytic current density remains low. Most probably, the size of the mesopores is too small to effectively trap FoDH1. The adsorbed FoDH1 catalyzes DET-type bioelectrocatalytic interconversion of the CO\(_2\)/HCOO\(^-\) and NAD\(^+\)/NADH redox couples. Most probably, one of the iron–sulfur clusters located near the enzyme surface communicates with mesoporous electrodes. When the communication proceeds effectively, FoDH1 behaves as a novel bidirectional catalyst for the substrates, since FoDH1 can realize fast uphill intramolecular electron transfer. The non-covalently bound flavin mononucleotide (FMN) cofactor in FoDH1 is dissociated from some FoDH1 molecules and adsorbs on the mesoporous electrode to give a symmetrical surface-confined redox wave. Although adsorbed FMN cannot participate in mediated electron transfer (MET)-type bioelectrocatalysis, dissociated FMN in solution works as a mediator for MET-type bioelectrocatalysis of the HCOO\(^-\) oxidation at planar electrodes.

1. Introduction

Oxidoreductase reactions are frequently coupled with electrochemical reactions. These coupled reactions are referred to as bioelectrocatalysis and are recognized as key reactions in the development of biosensors, bioreactors, and biofuel cells. They are also useful for understanding the kinetics and thermodynamics of redox enzyme reactions [1]. These reaction systems can be classified
into two categories depending on the electron transfer mode [2–8], i.e., direct electron transfer (DET) type, in which electron transfer occurs directly between enzymes and electrodes, and mediated electron transfer (MET) type, in which an artificial redox partner (mediator) shuttles electrons between enzymes and electrodes.

DET-type bioelectrocatalytic reactions are only observed with a limited number of redox enzymes and at limited kinds of electrodes suitable for individual redox enzymes. This is in part because the redox active centers of enzymes are often located deep within the polypeptide structure. It is well known that multi-copper oxidases, such as laccase [9,10], bilirubin oxidase [11–13], and Cu efflux oxidase [14,15], work well as DET-type bioelectrocatalysts for oxygen reduction. DET-type reactions have also been reported for oxidation of the first substrate with some membrane-bound redox enzymes, such as succinate dehydrogenase [16,17], alcohol dehydrogenase [18], gluconate dehydrogenase [19,20], hydrogenase [21,22], cellobiose dehydrogenase [23,24], and fructose dehydrogenase (FDH) [25–29].

Several authors have reported the use of nanostructures at electrode interfaces to increase the effective rate constant of long-range electron transfer by reducing the distance between the enzymes and the electrodes or by increasing the probability of productive orientation of the enzymes on the electrodes [30,31]. Previously, our group reported the effects of the pore size of mesoporous electrodes on the DET-type bioelectrocatalytic current of FDH from *Gluconobacter japonicas* (former name: *Gluconobacter frateurii*) [32,33]. The results indicated that catalytic current density increases with an increase in the pore size of the electrode, although the reason for this remains unclear. Furthermore, many authors have suggested that nanostructures on electrode surfaces improve contact with the buried active sites enough to electronically connect the enzyme with the electrode [34,35]. However, there is no solid evidence to support this idea. Many details of these DET-type redox reactions between enzymes and electrodes remain to be elucidated; however, it is clear that the performance of bioelectrochemical devices is enhanced by highly efficient and stable enzyme immobilization at electrodes modified with conductive nano-, micro-, or mesostructures [36].

Tungsten or molybdenum (W/Mo)-containing formate dehydrogenase (FoDH) is a vital enzyme for formate (HCOO\(^-\)) metabolism in bacteria and catalyzes the two-electron oxidation of HCOO\(^-\) to carbon dioxide (CO\(_2\)) (note that, strictly speaking, the term “CO\(_2\)” in this paper encompasses dissolved CO\(_2\), H\(_2\)CO\(_3\), HCO\(_3^-\), and CO\(_3^{2-}\) equilibrated in aqueous solutions). FoDH can work as a catalyst for bioanodes in enzymatic HCOO\(^-\)/O\(_2\) biofuel cells that are comparable to H\(_2\)/O\(_2\) biofuel cells in terms of the standard electromotive force [37]. It has been reported that some FoDHs can act as a CO\(_2\) reductase in the presence of a suitable electron donor [38–41]. Recently, FoDH from *Candida boidinii* has attracted significant attention as an electrocatalyst for biocathodes to reduce CO\(_2\) to HCOO\(^-\) at low overpotentials in artificial photosynthetic systems [42]. These enzymatic devices generally operate under mild conditions, i.e., ambient temperature and neutral pH.
In this study, we focus on the bioelectrocatalytic behavior of W-containing FoDH from *Methylobacterium extorquens* AM1 (FoDH1; EC 1.2.1.2) at planar and mesoporous carbon electrodes. FoDH1 is a heterodimeric soluble enzyme composed of two subunits (αβ) with molecular masses (mα) of 107 and 61 kDa, respectively. The α subunit contains the W-containing active site and at least one iron–sulfur (FeS) cluster. The β subunit contains non-covalently bound flavin mononucleotide (FMN) and an FeS cluster binding motif, and appears to be a fusion protein with its N-terminal domain related to NuoE-like subunits and its C-terminal domain related to NuoF-like subunits of well-known NADH–ubiquinone oxidoreductases (complex I) [43]. In addition, unlike many hydrogenases and other W/Mo-containing FoDHs, FoDH1 is not sensitive to O2 [43,44]. In a previous paper [45], we reported that FoDH1 does not produce a DET-type bioelectrocatalytic wave at planar carbon electrodes and requires suitable mediators to shuttle electrons between FoDH1 and electrodes. In addition, the electron transfer kinetics between FoDH1 and mediators (as artificial second substrates) obeys a linear free energy relationship (LFER), i.e., the enzyme exhibits no specificity to the second substrate, and the electron transfer rate constant between the enzyme and the second substrate increases exponentially with an increase in the driving force.

Herein, we provide a comprehensive study on the bioelectrocatalytic properties of FoDH1 focusing on the effects of mesoporous structures and on bidirectional bioelectrocatalysis. We have successfully observed DET-type bioelectrocatalysis of HCOO− oxidation and CO2 reduction at mesoporous electrodes. Redox communication between FoDH1 and the electrode was examined by monitoring DET-type bioelectrocatalysis of HCOO− oxidation and CO2 reduction at several carbon particle-modified glassy carbon electrodes. Bidirectional DET-type bioelectrocatalysis of NADH oxidation and NAD+ reduction was also examined at mesoporous electrodes. Consequently, we propose the importance of mesoporous structures to the DET-type bioelectrocatalysis of FoDH1 and rationalize the DET-type bioelectrocatalytic interconversion of the CO2/HCOO− and NAD+/NADH couples.

In addition, we have observed reversible redox waves with surface-confined FMN that is dissociated from FoDH1. The adsorbed FMN does not participate in MET-type bioelectrocatalysis of HCOO− oxidation at mesoporous electrodes. In contrast, a clear catalytic oxidation wave was observed in the presence of HCOO− at high concentration of FoDH1 at planar electrodes. This type of wave might be assigned to DET-type bioelectrocatalysis, but we have found that this wave is mediated by soluble FMN in solution dissociated from FoDH1.
2. Experimental

2.1 Materials

Ketjen Black EC300J (KB) with an average pore size of 39.5 nm was kindly donated by Lion Co. (Japan). Carbon cryogel (CCG) was synthesized using a method described in previous papers [32,33]. The mole ratio of resorcinol to catalyst (mol/mol) was 250 as appropriate and pH was adjusted to 10.0. The pore distribution of the CCG was evaluated from adsorption and desorption isotherms of N\textsubscript{2} at −196 °C using adsorption apparatus (BELSORP-mini II, BEL Japan Inc.). Fig. 1 shows the porous properties of the CCG in this study and the peak value of the pore radius was 39 nm. Poly (tetrafluoroethylene) fine powder (PTFE, 6-J) and poly (vinylidene difluoride) (PVDF) were obtained from DuPont Mitsui Fluorochemicals (Japan) and Kureha (Japan), respectively. A miller-like (and apparently flat) glassy carbon electrode (GCE, 3 mm in diameter) was obtained from BAS Inc. (USA). FMN was obtained from Sigma Aldrich Co. (USA). Methyl viologen dichloride (MV) was obtained from Tokyo Chemical Industry (Japan). FoDH1 was purified according to the literature [45]. All other chemicals used in this study were obtained from Wako Pure Chemical (Japan) and were of analytical grade. All solutions were prepared with distilled water.

Figure 1.

Pore-size distribution of the synthesized CCG
2.2 Preparation of electrodes

KB- and CCG-modified GCEs (KB/GCEs and CCG/GCEs) were prepared according to a method described in a previous paper [46] using a KB:PTFE or CCG:PVDF ratio of 4:1 (w/w). Anodized GCEs (AGCEs) were prepared according to the literature [47]. The projective surface area of all GCEs was 0.071 cm$^2$.

2.3 Electrochemical measurements

All electrochemical measurements were performed with a BAS CV-50W electrochemical analyzer. A handmade Ag|AgCl|KCl (sat.) electrode and a Pt wire were used as the reference and counter electrodes, respectively. All the potentials reported here are referred to the reference electrode. Measurements were carried out in a 0.1 M potassium phosphate buffer at pH 7.0 and 25 °C (M = mol dm$^{-3}$).

2.4 Quartz crystal microbalance (QCM) with KB-modified electrodes

QCM measurements were performed using a Seiko EG&G QCA917 QCM analyzer at room temperature. 9-MHz At-cut quartz crystal plates coated with Au (Seiko EG&G Co., Ltd.) were used as resonators, of which the projected surface area was 0.196 cm$^2$. The KB slurry prepared in Section 2.2 (6 μL) was applied on the resonator and dried at room temperature to prepare a KB-modified quartz crystal resonator (L = dm$^{-3}$). In some cases, the QCM resonator was also used as a working electrode for electrochemical QCM (EQCM).

2.5 FoDH1 assay

The FoDH1 activity was measured spectrophotometrically with 1-methoxy-5-methylphenazinium (PMS) and 2,6-dichlorophenolindophenol sodium salt hydrate (DCIP) as described previously [45]. One unit of the FoDH1 activity is defined as the amount of FoDH1 that catalyzes the reduction of 1 μmol of DCIP per min.
3. Results and discussion

3.1 Electrochemistry of FoDH1 adsorbed on KB/GCEs

The solid line in Fig. 2A shows the cyclic voltammogram (CV) of 20 μM FoDH1 at KB/GCE in 0.1 M phosphate buffer (pH 7.0). Peak-shaped anodic and cathodic waves appear at a peak potential ($E_p$) of $-0.43$ V. These waves are practically symmetrical, and the peak height ($i_p$) increases proportionally with the scan rate ($v$) up to at least $v = 100$ mV s$^{-1}$ (data not shown). Therefore, the waves are assigned to a surface-confined species. The decrease in the non-Faradaic background current after the addition of FoDH1 is due to the adsorption phenomena. The $E_p$ value is in good agreement with that recorded for free FMN adsorbed on the electrode in separate experiments. The half-peak width of the adsorption wave is 40 mV, which is close to the theoretical value for the reversible one-step two-electron process (90.6/2 mV).

The formal potential ($E^{\circ'}$) of the cofactor buried in the enzyme is generally not consistent with that of the cofactor in the free state [48–54]. For example, $E^{\circ'}$ of FMN in complex I is 0.133 V more negative than that of free FMN [55]. The structure and function of the β subunit of FoDH1 are considered to be similar to those of the flavoprotein sub-complex of complex I [42]. Thus, it may be assumed that $E^{\circ'}$ for non-covalently bound FMN in FoDH1 is also more negative than that of free FMN. Therefore, the symmetrical redox signal may be assigned to free FMN that is dissociated from

![Graph A](image.png)

![Graph B](image.png)

**Figure. 2**

(A) CV of FoDH1 (20 μM) in 0.1 M phosphate buffer (pH 7.0) at KB/GCE 170 min after the addition of FoDH1 (solid line). The dotted line is a background CV (before the addition of FoDH1). The data were taken at $v = 10$ mV s$^{-1}$.

(B) Time dependence of the anodic peak current density ($j_{pa}$) at KB/GCE under quiescent conditions.
FoDH1 and adsorbed on the electrode. The peak heights of the waves gradually increase with an increase in exposure time (Fig. 2B). Note that during the experiments, the electrode and solution were kept in the dark to avoid the photolytic reaction of the flavins [56]. The surface concentration of free FMN ($J_{\text{FMN}}$) is evaluated to be $(2 \pm 1) \times 10^{-10}$ mol cm$^{-2}$ from the area of the background-subtracted CV recorded 6 h after the addition of FoDH1 (1.5 μM).

Upon the addition of sodium formate (HCOONa) to the FoDH1-containing solution, a steady-state oxidation wave appears, together with the surface-confined wave of free FMN, as shown in Fig. 3A. The steady-state wave is assigned to the HCOO$^-$ oxidation catalyzed by FoDH1 adsorbed on KB/GCE. The shape of the surface-confined wave of free FMN remains unchanged, indicating that free FMN adsorbed on KB/GCE does not work as a mediator between adsorbed FoDH1 and the electrode. FMN may adsorb on the electrode with the hydrophobic isoalloxazine ring oriented parallel to the electrode surface and the hydrophilic adenine side chain directed to the solution side [57]. The redox active isoalloxazine ring may be thus shielded by the side chain to prevent contact and redox reaction with FoDH1. Even when we added free FMN to the experimental system, only the surface-confined peak increased and any MET-type catalytic current and any diffusion-controlled faradaic wave of free FMN in solution were not observed (Fig. 4A). The result indicates that almost all FMN are immobilized on KB/GCEs and no free FMN exists in the solution. This seems to be due to a large surface area of KB/GCE. On the other hand, when we added MV as a hydrophilic mediator, a clear MET-type catalytic wave was observed (Fig. 4B). Therefore, a free soluble mediator can work with FoDH1-adsorbed KB/GCEs.
In addition, the oxidation current density increases slowly with exposure time under quiescent conditions and reaches a maximum value of 0.4 mA cm$^{-2}$ (Fig. 3B). A similar time-dependent increase in the catalytic current density has been reported for the DET-type bioelectrocatalysis of FDH at KB-modified electrodes [58]. Almost identical CVs are obtained when KB/GCE used in the above experiments is transferred to a fresh solution of HCOO$^-$ in the absence of FoDH1, as shown in Fig. 5. Therefore, we can conclude that FoDH1 absorbs slowly on KB/GCEs and

![Figure 3](image_url)

**Figure 3**

(A) CV of FoDH1 (20 μM) in 0.1 M phosphate buffer (pH 7.0) in the presence of 50 mM HCOONa at KB/GCE 170 min after the addition of FoDH1 (solid line). The dotted line is a background CV (in the absence of FoDH1 and HCOONa). The data were taken at $v = 10$ mV s$^{-1}$.

(B) Time dependence of the steady-state limiting current density ($j$ at $-0.2$ V) for the FoDH1-catalyzed HCOO$^-$ oxidation (50 mM) at KB/GCE under quiescent conditions. The current includes the background because the latter must decrease during the adsorption.

(C) CV of FoDH1 (20 μM) in 0.1 M phosphate buffer (pH 6.6) in the presence of 50 mM CO$_2$ at KB/GCE 170 min after the addition of FoDH1 (solid line). The dotted line is a background CV (in the absence of FoDH1 and CO$_2$). The data were taken at $v = 10$ mV s$^{-1}$.

(D) Time dependence of the resonance frequency shift ($Δf$) in QCM measurements. FoDH1 was added at a final concentration of 1.5 μM into 0.1 M phosphate buffer (pH 7.0) at a point indicated by the arrow.
works as a DET-type bioelectrocatalyst.

Conversely, in the presence of CO$_2$ (by the addition of sodium hydrogen carbonate), a steady-state wave owing to CO$_2$ reduction catalyzed by FoDH1 adsorbed on the electrode is observed (Fig. 3C). The maximum current density is $-0.3$ mA cm$^{-2}$. The broad anodic wave around $-0.4$ V, which is different from the adsorption waves of free FMN, is assigned to the catalytic oxidation of HCOO$^-$ generated near the electrode surface in a potential region more negative than $-0.6$ V. On the other hand, FMN-adsorbed KB/GCEs did not give a clear signal of the electrocatalysis in the presence of HCOO$^-$ and CO$_2$, as shown in Fig. 6. Therefore, adsorbed FoDH1 can work as a DET-type bioelectrocatalyst for the interconversion of HCOO$^-$ and CO$_2$ at KB/GCEs. The zero-current potential (ca. $-0.58$ V) must correspond to the equilibrium potential of CO$_2$/HCOO$^-$ ($E_{CO_2}^{eq}$):

$$E_{CO_2}^{eq} = E_{CO_2}^{\text{ref}} + \frac{RT}{2F} \ln \frac{c_{CO_2}}{c_{HCOO^-}} ,$$

where $R$ is the gas constant, $T$ is the absolute temperature, $F$ is the Faraday constant, $E_{CO_2}^{\text{ref}}$ is the equilibrium potential of CO$_2$/HCOO$^-$.

![Figure 4](image_url)

**Figure 4.**
(A) CVs of FoDH1 (20 μM) in 0.1 M phosphate buffer (pH 7.0) in the presence of 50 mM HCOONa at KB/GCE 170 min after the addition of FoDH1 (dashed line) at 30 °C, and 60 min after the addition of 400 μM FMN (solid line). The dotted line is a background CV (in the absence of FoDH1, HCOONa, and FMN). The scan rate is 10 mV s$^{-1}$.
(B) CVs of FoDH1 (7.6 μM) in 0.1 M phosphate buffer (pH 7.5) in the presence of 50 mM HCOONa and at KB/GCE 170 min after the addition of FoDH1 (dashed line) at 30 °C, and in the presence of 400 μM MV (solid line). The dotted line is a background CV (in the absence of FoDH1 and HCOONa). The scan rate is 10 mV s$^{-1}$.
formal potential at pH 6.6 (−0.61 V [59]), and $c_{\text{CO}_2}$ and $c_{\text{HCOO}^-}$ are the concentrations of CO$_2$ and HCOO$^-$ near the electrode surface, respectively (in this case, $c_{\text{HCOO}^-}$ is estimated to be about 5 mM).

The DET-type bioelectrocatalytic activity of FoDH1 is qualitatively similar to those exhibited by W-containing FoDH from *Syntrophobacter fumaroxidans* on a graphite–epoxy electrode [60] and Mo-containing FoDH from *Escherichia coli* on a pyrolytic graphite electrode [61]. The effect of the structure of the electrodes on such DET-type bioelectrocatalysis is described later.

We also performed QCM measurements to monitor FoDH1 adsorption under quiescent conditions (Fig. 3D). In this study, we prepared a resonator modified with KB. The surface concentration ($\Gamma_t$) was evaluated as $(8 \pm 4) \times 10^{-9}$ mol cm$^{-2}$ calculated from the frequency shift ($\Delta f$) 2.5 × 10$^4$ s after the addition of FoDH1 at 1.5 μM using the Sauerbrey equation [62]:

$$\Delta f = -\frac{2f_0^2 \Delta m}{A \sqrt{\mu_q \rho_q}},$$

(2)

where $f_0$ is the fundamental resonance frequency (9 MHz), $\Delta m$ is the mass change on the electrode, $A$ is the surface area, $\mu_q$ is the shear module ($2.947 \times 10^{10}$ kg m$^{-1}$ s$^{-2}$), and $\rho_q$ is the density of quartz ($2.648 \times 10^3$ kg m$^{-3}$). The $\Gamma_t$ value is much larger than $\Gamma_{\text{FMN}}$ electrochemically evaluated in the above.

**Figure 5.**

CVs of FoDH1 (20 μM) in 0.1 M phosphate buffer (pH 7.0) in the presence of 50 mM HCOONa at KB/GCE 170 min after the addition of FoDH1 (dotted line) at 30 °C, and after transferring the FoDH1-adsorbed KB/GCE to fresh 0.1 M phosphate buffer (pH 7.0) in the absence (dashed line) and in the presence (solid line) of 50 mM HCOONa. The scan rate is 10 mV s$^{-1}$. 
Therefore, we conclude that both released FMN and FoDH1 are independently absorbed on KB/GCEs.

Similar DET-type bioelectrocatalytic behavior is observed at a CCG/GCE (Fig. 7A), but not at a bare planar GCE (Fig. 7B). Even after anodizing bare GCE to produce AGCE, where anodization of GCE generates many nanometer-size pits and increases its hydrophilicity [47], no DET-type bioelectrocatalytic wave is observed, indicating that the pits are too small to trap FoDH1. Thus, pore size is a more important factor than surface hydrophilicity or hydrophobicity for the direct

![Figure 6](image1)

**Figure 6.**
CVs in 0.1 M phosphate buffer (pH 7.0) 60 min after the addition of 20 μM FMN in the absence (dashed line) and in the presence of 50 mM HCOONa and CO₂ (A, solid line) or 30 mM NAD⁺ and NADH (B, solid line) at 30 °C. The dotted line is a background CV (in the absence of FMN and CO₂/HCOONa or NAD⁺/NADH).

![Figure 7](image2)

**Figure 7.**
CVs of FoDH1 (20 μM) in 0.1 M phosphate buffer (pH 7.0) in the presence of 50 mM HCOONa at CCG/GCE (A) and AGCE (B) 170 min after the addition of FoDH1 (solid line) at 30 °C. The dotted line is a background CV (in the absence of FoDH1 and HCOONa). The scan rate is 10 mV s⁻¹.
communication of FoDH1 with electrodes. Furthermore, some pore-related curvature effects may increase the number of enzymes with orientations suitable for electron transfer.

It is interesting to note that such bidirectional DET-type bioelectrocatalysis is also observed for membrane-bound hydrogenase from Desulfovibrio vulgaris Miyazaki F (\(m_u\): 98 kDa [63]) at KB-modified carbon electrodes [64], but that the current density of the hydrogenase is much larger than that of FoDH1. Thus, the size of the mesopores in the electrodes used here appears to be slightly too small to effectively trap FoDH1. Consequently, we are currently attempting to synthesize new materials with larger mesopores suitable for trapping FoDH1.

3.2 NADH oxidation and NAD\(^+\) reduction catalyzed by FoDH1 absorbed on KB/GCEs

The \(\beta\) subunit of FoDH1 shows diaphorase-like activity in the oxidation of NADH to NAD\(^+\) when coupled with reduction by certain electron acceptors [65]. Therefore, we attempted to detect a DET-type catalytic wave from NADH oxidation and NAD\(^+\) reduction. Figure 8 shows the CV at FoDH1-adsorbed KB/GCE in the presence of NADH and NAD\(^+\). Clear steady-state oxidation and reduction waves are observed at potentials more positive than \(-0.4\) V and more negative than \(-0.6\) V, respectively, and are ascribed to NADH oxidation and NAD\(^+\) reduction catalyzed by FoDH1 adsorbed on KB/GCE, respectively. The zero-current potential (\(-0.52\) V) is close to the equilibrium potential of the NAD\(^+\)/NADH couple (\(E_{\text{NAD}}^{\text{eq}}\)):

\[
E_{\text{NAD}}^{\text{eq}} = E_{\text{NAD}}^{\circ} + \frac{RT}{2F}\ln\frac{c_{\text{NAD}^+}}{c_{\text{NAD}}} 
\]

(3)

where \(E_{\text{NAD}}^{\circ}\) is the formal potential of the NAD\(^+\)/NADH couple (\(-0.517\) V at pH 7.0 [66]), and \(c_{\text{NAD}^+}\) and \(c_{\text{NAD}}\) are the concentrations of NAD\(^+\) and NADH near the electrode surface, respectively (in this case, \(E_{\text{NAD}}^{\text{eq}} = E_{\text{NAD}}^{\circ}\)). This means that FoDH1 catalyzes the DET-type bioelectrocatalytic interconversion of both the first and second substrates, i.e., the CO\(_2\)/HCOO\(^-\) and NAD\(^+\)/NADH couples. FMN-adsorbed KB/GCEs (in the absence of FoDH1) also did not give any catalytic wave in the presence of the NAD\(^+\)/NADH couple (Fig. 6B).

While Mo/W-containing FoDHs show large diversity in their subunit composition and cofactor binding, their active sites are highly similar [44]. In the solution reaction of FoDH1, HCOO\(^-\) as the first reduced substrate is oxidized at the W-containing catalytic center [43]. The electrons are transferred to bound FMN (as a one/two-electron converter) via the FeS clusters and finally to NAD\(^+\) in solution in a mechanism similar to that of the flavoprotein sub-complex of complex I [67]. In the
reverse reaction, the second reduced substrate (NADH) is oxidized at FMN, and the electrons are transferred to the W-center via the FeS clusters and finally to CO$_2$ in solution. In this DET-type bioelectrocatalysis, it is likely that one of the FeS clusters located near the surface of FoDH1 communicates directly with the mesoporous electrode.

In the interconversions of the CO$_2$/HCOO$^-$ and NAD$^+$/NADH couples, at least one uphill intramolecular electron transfer process with a positive standard Gibbs energy ($\Delta_rG^0 > 0$) must occur. Considering the fact that FoDH1 can catalyze the bidirectional DET-type bioelectrocatalysis of the CO$_2$/HCOO$^-$ and NAD$^+$/NADH couples, and that $E^\circ_{\text{CO}_2}$ is 0.11 V more negative than $E^\circ_{\text{NAD}^+}$, the intramolecular uphill electron transfer process(es) must proceed very rapidly. This means that the rate constants of the self-electron-exchange reactions at the redox sites in the enzyme as electron-donating and -accepting sites ($k_{\text{DD}}$ and $k_{\text{AA}}$) are very large [68]:

$$k \approx \sqrt{k_{\text{DD}}k_{\text{AA}} \exp \left( -\frac{\Delta_rG^0}{RT} \right)} > 1,$$  \hspace{1cm} (4)

where $k$ is the rate constant for the FoDH1 reaction. In addition, considering the fact that the zero-current potential of the bidirectional catalytic wave corresponds to the equilibrium potential of the substrates ($E_{\text{CO}_2}^{\text{eq}}$ and $E_{\text{NAD}^+}^{\text{eq}}$), the rate constant of the interfacial electron transfer between the substrates ($E_{\text{CO}_2}^{\text{eq}}$ and $E_{\text{NAD}^+}^{\text{eq}}$), the rate constant of the interfacial electron transfer between the

![Figure 8.](image)

CV at the FoDH1-adsorbed KB/GCE in 0.1 M phosphate buffer (pH 7.0) containing 10 mM NAD$^+$ and 10 mM NADH (solid line). The dotted line represents a background CV before the addition of FoDH1. The data were taken at $v = 10$ mV s$^{-1}$. FoDH1 was adsorbed from 20 $\mu$M FoDH1 for 170 min under quiescent conditions.
expected electron-donating/accepting site (an FeS cluster) in FoDH1 and the mesoporous electrode is
large when FoDH1 adsorbs with a productive orientation. Under such conditions, FoDH1 behaves as
a novel reversible catalyst with an apparent formal potential close to that of the substrates.

3.3 MET-type bioelectrocatalysis of FoDH1 without added mediators

As described above, FoDH1 cannot directly communicate with planar electrodes at rate
constants sufficient to produce clear DET-type bioelectrocatalytic waves. At high concentration of
FoDH1 (20 ± 1 μM), however, a steady-state wave is observed without addition of any mediators in
the presence of HCOO−, as shown in Fig. 9. One may assign this wave to DET-type bioelectrocatalysis.
However, the half-wave potential ($E_{1/2}$) of the catalytic wave is practically in good agreement with the
formal potential of free FMN in solution ($E^o_{FMN} = -0.41$ V at pH 7.0 [66]). In addition, no time
dependence of the wave (as observed in the DET-type bioelectrocatalysis, Fig. 3B) is observed in this
case. Upon the addition of free FMN (17 μM), the catalytic current increases without a change in $E_{1/2}$
(Fig. 9). A similar bioelectrocatalytic oxidation wave is observed at gold and indium tin oxide
electrodes. Therefore, the steady-state wave may be ascribed to free FMN-mediated catalytic oxidation
of HCOO−.

We attempted to make sure the reconstruction (generation of holo-enzyme from apo-enzyme
on the addition of free FMN). Addition of FMN to the FoDH1 solution, the enzyme activity in solution

![Figure 9] Figure 9.
CVs of FoDH1 (20 μM) at a planar GCE in 0.1 M phosphate buffer (pH 7.0) in the absence
(dotted line) and presence (solid line) of 50 mM HCOONa and 50 mM HCOONa, and after
the addition of 17 μM FMN (broken line). The data were taken at $v = 10$ mV s$^{-1}$. 

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increased slightly (Fig. 10). The reconstruction effect was also checked in MET-type bioelectrocatalysis of FoDH1 with MV as a soluble mediator. The MET-type catalytic current increased slightly on the addition of free FMN in the presence of HCOO\(^-\) (Fig. 10B, C). The result shows that the dissociation of FMN from FoDH1 is in equilibrium and the addition of FMN slightly increases the concentration of holo-FoDH1. Therefore, the increase in the MET-type catalytic current on the addition of FMN in Fig. 9 is ascribed in part to the reconstruction of holo-FoDH1, but predominantly to the increase in the concentration of free soluble FMN in solution as a mediator.

It is noted here that LFER is responsible for the electron transfer kinetics between FoDH1 and the mediator for both HCOO\(^-\) oxidation and CO\(_2\) reduction, and that bidirectional MET-type bioelectrocatalysis occurs only when a mediator with a formal potential close to \(E^\circ_{\text{CO}_2}\) is used \([45]\). These characteristics indicate that a non-specific electron transfer occurs between an electron-donating/accepting site in FoDH1 and the mediator. This site may be identified as FeS clusters near the enzyme surface, as with the case of DET-type bioelectrocatalysis.

In the free FMN-mediated system, no catalytic wave for CO\(_2\) reduction is observed. This is because \(E^\circ_{\text{FMN}}\) is more positive than the formal potential of the FeS cluster site near the enzyme surface, and such an uphill electron transfer proceeds with difficulty in solution.
Figure 10.

(A) Comparison of the FoDH1 activity with adding 1 μM FMN into the solution and without adding it. FoDH1 activity was measured at pH 7.0. The error bars were evaluated by the Student t-distribution at a 90% confidence level.

(B) CVs recorded on a GCE in 0.1 M phosphate buffer (pH 7.0) containing 0.1 mM MV and 30 mM HCOO⁻ in the absence (dash line) and presence (dotted line) of 1.2 μM FoDH1. Solid line is a CV recorded after adding FMN at a final concentration of 19 μM into the solution. The scan rate is 10 mV s⁻¹.

(C) Chronoamperometry at −0.5 V of FoDH1 (1.2 μM) at a GCE in 0.1 M phosphate buffer (pH 7.0) in the presence of 30 mM formate and 0.1 mM MV. FMN was added at a final concentration of 19 μM into the solution at the point indicated by the arrow. (A) CVs of FoDH1 (20 μM) at a planar GCE in 0.1 M phosphate buffer (pH 7.0) in the absence (dotted line) and presence (solid line) of 50 mM HCOONa and 50 mM HCOONa, and after the addition of 17 μM FMN (broken line). The data were taken at v = 10 mV s⁻¹.
4. Conclusions

FoDH1 adsorbed on mesoporous electrodes realizes DET-type bioelectrocatalytic interconversion of the \( \text{CO}_2/\text{HCOO}^- \) redox couple with \( E^{\circ}_{\text{CO}_2} = -0.63 \) V (pH 7.0) and of the \( \text{NAD}^+/\text{NADH} \) redox couple with \( E^{\circ}_{\text{NAD}} = -0.517 \) V (pH 7.0). Most probably, one of the FeS clusters located near the FoDH1 surface communicates with the porous electrodes. The formal potential of the FeS cluster seems to be located between \( E^{\circ}_{\text{CO}_2} \) and \( E^{\circ}_{\text{NAD}} \). The occurrence of this bidirectional bioelectrocatalysis supports the idea that interfacial electron transfer proceeds with a relatively large rate constant, and that FoDH1 realizes fast uphill intramolecular electron transfer, most probably due to the large rate constants of the self-electron exchange reactions at the redox sites in FoDH1.

This study also revealed that the non-covalently bound FMN cofactor dissociates from some FoDH1 molecules and adsorbs on mesoporous electrodes to give a symmetrical surface-confined redox wave. Although the adsorbed FMN cannot participate in MET-type bioelectrocatalysis of FoDH1, the dissociated FMN in solution works as a mediator in the MET-type bioelectrocatalysis of HCOO\(^-\) oxidation catalyzed by FoDH1 in solution at planar electrodes. The latter electrochemical phenomena may be misinterpreted as FoDH1 causing DET-type bioelectrocatalysis at planar electrodes.

There are many reports of DET-type bioelectrocatalysis, including those occurring with FAD-dependent glucose oxidase (GOD) [47,69–93] and PQQ-dependent soluble glucose dehydrogenase (sGDH) [94–99]. However, some of these reports seem to present disputable conclusions. FAD in GOD is also non-covalently bound to the enzyme and may release from holo-GOD. In addition, it is well known that PQQ dissociates easily from the active center of holo-sGDH [100]. Therefore, careful examination of catalytic waves (including those reported in the literature) is required for the identification of DET- and MET-type bioelectrocatalysis without added mediators.

5. References

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1-2. Direct electron transfer-type four-way bioelectrocatalysis of CO₂/formate and NAD⁺/NADH redox couples by tungsten-containing formate dehydrogenase adsorbed on gold nanoparticle-embedded mesoporous carbon electrodes modified with 4-mercaptopyridine

Abstract
Tungsten-containing formate dehydrogenase from Methylobacterium extorquens AM1 (FoDH1) catalyzes formate oxidation with NAD⁺. FoDH1 shows little direct communication with carbon electrodes, including mesoporous Ketjen Black-modified glassy carbon electrode (KB/GCE); however, it shows well-defined direct electron transfer (DET)-type bioelectrocatalysis of carbon dioxide reduction, formate oxidation, NAD⁺ reduction, and NADH oxidation on gold nanoparticle (AuNP)-embedded KB/GCE treated with 4-mercaptopyridine. Microscopic measurements reveal that the AuNPs ($d = 5$ nm) embedded on the KB surface are uniformly dispersed. Electrochemical data indicate that the pyridine moiety on the AuNPs plays important roles in facilitating the interfacial electron transfer kinetics and increasing the probability of productive orientation of FoDH1. The formal potential of the electrochemical communication site, which is most probably an ion-sulfur cluster, is evaluated as $-0.591 \pm 0.005$ V vs. Ag/AgCl|sat. KCl from Nernst analysis of the steady-state catalytic waves.

1. Introduction

Bioelectrocatalysis, in which an enzyme reaction is coupled with an electrode reaction, is an essential process for biofuel cells, biosensors, and bioreactors [1–10]. It is classified in two types: mediated electron transfer (MET) and direct electron transfer (DET). The DET-type reaction is an ideal process because it can provide simple systems in which electrodes are modified only with enzymes. It is also possible to minimize the thermodynamic overpotential required for electron transfer between an enzyme and an electrode. For a rapid DET-type reaction, it is essential that one of the redox sites is located near the enzyme surface and that the enzymes are suitably oriented on the electrode surface to minimize the distance between the redox site and the electrode surface [11–14].

Recent progress has been reported in the analysis and improvement of the characteristics of DET-type bioelectrocatalysis [4,15]. An important issue in this field is the use of mesoporous electrodes as platforms for DET-type bioelectrocatalysis. The curvature effect of mesoporous structures appears to increase the probability of productive orientations suitable for DET-type reactions [16,17]. Gold nanoparticles (AuNPs) are frequently utilized in DET-type bioelectrocatalysis. AuNPs, which have a variety of sizes and electronic properties, are expected to provide versatile building blocks for DET-type bioelectrocatalysis; also, the surface properties of AuNPs can be readily
controlled and functionalized with thiols. It has been reported that the utilization of AuNPs improves long range electron transfer kinetics for various proteins, including heme proteins such as membrane cytochrome c oxidase [18–21], the blue copper protein azurin, [22], and sulfite oxidase [23]. Porous three-dimensional networks of AuNPs obtained by drop casting of concentrated gold colloids were shown to enhance DET-type bioelectrocatalysis of hydrogenase [24], bilirubin oxidase [25,26], laccase [27–30], and cellobiose dehydrogenase [31]. In addition, sugar/dioxygen (O2) [32–34] and hydrogen/O2 biofuel cells [24] have been constructed with AuNP-based bioanodes or biocathodes.

Tungsten (W)-containing formate dehydrogenase from Methylobacterium extorquens AM1 (FoDH1) is a heterodimeric enzyme with a molecular mass of approximately 170 kDa [35]. This enzyme possesses a W-containing catalytic site (Wc), non-covalently bound flavin mononucleotide (FMN), and iron sulfur clusters; it catalyzes formate (HCOO−) oxidation with NAD+ [35]. FoDH1 is a promising MET-type bioelectrocatalyst for the oxidation of HCOO− to carbon dioxide (CO2), the reduction of CO2, the oxidation of NADH, and the reduction of NAD+ [36]. We constructed a high-power MET-type HCOO−/O2 biofuel cell with a maximum power density of 12 mW cm−2 and an open circuit potential of 1.2 V under quiescent conditions [37] as well as an efficient MET-type gas-diffusion bioelectrocatalytic CO2 reduction system at a current density of 17 mA cm−2 under mild conditions [38]. Although FoDH1 does not communicate directly with flat electrodes, it provides very small DET-type bioelectrocatalytic signals upon the interconversion of the CO2/HCOO− and NAD+/NADH redox couples at mesoporous Ketjen Black-modified glassy carbon electrode (KB/GCE) [39]. This four-way bioelectrocatalytic reaction is unique. However, detailed analysis of the current-potential curves was difficult because the catalytic current density was very small and no clear sigmoidal response was observed.

In this work, we constructed a new platform for the DET-type bioelectrocatalysis of FoDH1. We developed AuNP-embedded KB/GCE treated with 4-mercaptopyridine to generate a pyridine-terminal monolayer on the surface of the AuNP. We investigated the role of the pyridine-terminal monolayer in the interaction between FoDH1 and the electrode and analyzed the steady-state bioelectrocatalytic waves to understand the DET-type bioelectrocatalysis.

2. Experimental

2.1. Chemicals

Ketjen Black (KB, EC300J) was kindly donated by Lion Corp. (Japan). Poly(1,1,2,2-tetrafluoroethylene) (PTFE, 6-J) fine powder was purchased from DuPont-Mitsui Fluorochemicals Co., Ltd. (Japan). A colloid of AuNPs 5 nm in diameter was obtained from BBI Solutions (UK). 4-Mercaptopyridine was obtained from Sigma-Aldrich Co. LLC (USA). Other chemicals were obtained
from Wako Pure Chemical Ind., Ltd. (Japan), and all solutions were prepared with distilled water. FoDH1 was purified according to a literature procedure [36].

2.2. Electrode preparation

KB powder (40 mg) was mixed with PTFE (10 mg) and homogenized in 3.5 mL of 2-propanol for 3 min at 0 °C to prepare a KB slurry ($L = \text{dm}^3$). A 3 µL aliquot of the KB slurry was applied on glassy carbon electrode (GCE, 3 mm in diameter, BAS, USA) to prepare KB/GCE. A 30 µL aliquot of the AuNP colloid was cast on the surface of the KB/GCE and dried under reduced pressure. This procedure was repeated three times. This electrode is referred to as AuNP/KB/GCE. AuNP/KB/GCE was exposed to 4-mercaptopyridine vapor to create pyridine-terminated groups on the AuNPs. The reaction time was 1 min at 280 °C. This electrode is referred to as Py/AuNP/KB/GCE. Finally, FoDH1 was immobilized on Py/AuNP/KB/GCE by immersing the electrode in 40 µL of an FoDH1 solution (15 mg mL$^{-1}$ of FoDH1 and 250 µM sodium formate in 0.1 M phosphate buffer with pH 6.0) for 10 min at 4 °C ($M = \text{mol dm}^{-3}$).

2.3. Microscopic and electrochemical measurement procedures

Transmission electron microscopy (TEM) was performed with a JEM-2200FS field emission electron microscope (JEOL, Japan) operated at 200 kV. To prepare the TEM sample, AuNP-modified KB powder was exfoliated from the surface of AuNP/KB/GCE and placed on a mesh copper grid carbon film.

Cyclic voltammetry and chronoamperometry were conducted on an ALS 611B electrochemical analyzer (BAS, USA). A Pt wire and a homemade Ag|AgCl|sat. KCl electrode was used as the counter and reference electrodes, respectively. All potentials in this study are given with respect to the reference electrode. The measurements were performed in 1.0 M phosphate buffer with various pH values at 25 °C under quiescent conditions (without any forced convection).

3. Results and Discussion

3.1 Characterization of AuNP/KB/GCEs

The cyclic voltammogram (CV) of AuNP/KB/GCE obtained in 0.5 M H$_2$SO$_4$ showed a reducing peak at 0.9 V (data not shown); this is assigned to the reduction of Au oxide on the electrode [40]. The TEM image of the AuNP/KB/GCE surface (Fig. 1A) indicates that the KB particles range from 30 to 40 nm in diameter and that AuNPs approximately 5 nm in diameter are uniformly dispersed
on the KB surface without aggregation. These results confirm the successful embedding of AuNPs on the KB surfaces and that the mesoporous three-dimensional structures are formed not by AuNPs but

![Figure 1](image-url)

**Figure 1.**
(A) TEM image of AuNP-modified KB powder.
(B) CVs for HCOO⁻ oxidation at FoDH1-modified Py/AuNP/KB/GCE (1, red solid line) and FoDH1-modified KB/GCE (2, blue solid line) in 1.0 M phosphate buffer (pH 8.0) containing 500 mM sodium formate under Ar-saturated and quiescent conditions at 25 °C. The scan rate (υ) was 10 mV s⁻¹. The dotted lines (1 bg and 2 bg) represent the CVs in the absence of substrate, respectively. The broken line (1 rs) shows the expected residual slope. The inset shows the Nernst analysis of the sigmoidal curve of the catalytic wave at FoDH1-modified Py/AuNP/KB/GCE, obtained by subtracting curve 1 rs from curve 1. The normalized and subtracted voltammetric data are indicated by open circles. The solid curve indicates the refined curve obtained by non-linear regression analysis based on Eq. (1).
3.2. DET-type bioelectrocatalysis at FoDH1-adsorbed Py/AuNP/KB/GCEs

Figure 1B shows CVs at FoDH1-adsorbed Py/AuNP/KB/GCE at pH 8.0. A clear steady-state wave is observed in the presence of 0.5 M HCOO⁻ (curve 1). The wave is assigned to HCOO⁻ oxidation catalyzed by FoDH1 adsorbed on Py/AuNP/KB/GCE. The sigmoidal curve at approximately −0.6 V is followed by a straight and gradual increase of the current with increasing electrode potential. The increasing part is called the residual slope; it is caused by slow heterogeneous electron transfer of randomly and unfavorably orientated enzymes [4]. A similar catalytic wave was observed at FoDH1-adsorbed KB/GCE; however, this wave was very small and contained only a residual slope without a sigmoidal curve (curve 2).

When FoDH1 was adsorbed on AuNP/KB/GCE without 4-mercaptopyridine treatment, the wave was similar to that observed at KB/GCE. In addition, the large catalytic wave at FoDH1-adsorbed Py/AuNP/KB/GCE disappeared after reductive desorption of 4-mercaptopyridine at −1.2 V, while the small catalytic wave at FoDH1-adsorbed AuNP/KB/GCE remained unchanged at −1.2 V. Contrastingly, when FoDH1 was adsorbed on a 4-mercaptopyridine-treated planar gold electrode, no DET-type catalytic wave was observed. Therefore, the pyridine moiety on the surface of the mesoporous electrode is essential to realize large sigmoidal DET-type catalytic waves. Most probably, the pyridine moiety plays important roles in increasing the probability of productive orientation and improving the heterogeneous electron transfer kinetics.

We attempted to characterize the sigmoidal part of the steady-state catalytic wave observed at FoDH1-adsorbed Py/AuNP/KB/GCE. The contribution from the slow heterogeneous electron transfer of unfavorably orientated FoDH1 was eliminated by subtracting the expected residual slope according to the literature [41], as indicated by a broken line in Fig. 1B. The following Nernst-type equation was fitted to the sigmoidal curve by non-linear least squares analysis using the Gnuplot graphing utility:

\[
\frac{j_s(E)}{j_{s,\text{lim,ox}}} = \frac{\eta}{1 + \eta}
\]

(1)

where \(j_s(E)\) and \(j_{s,\text{lim,ox}}\) are the steady-state current density at a potential \((E)\) and the steady-state limiting oxidation current density, respectively, and \(\eta\) is defined by

\[
\eta = \exp\left(\frac{n_{\text{app}} F (E - E_{\text{ox}}^\circ)}{RT}\right)
\]

(2)

where \(n_{\text{app}}\) and \(E_{\text{ox}}^\circ\) are the apparent number of the electron in the heterogeneous electron transfer and the formal potential of the electrochemical communication site of an enzyme, respectively. These were
used as adjusting parameters in the non-linear least-square analysis. Therefore, $n_{app}$ includes the kinetic factor. $F$, $R$, and $T$ are the Faraday constant, gas constant, and absolute temperature, respectively. The

\[ \text{Figure 2.} \]

(A) CVs for HCOO$^-$ oxidation and CO$_2$ reduction at FoDH1-modified Py/AuNP/KB/GCE in 1.0 M phosphate buffer (pH 6.6) containing 10 mM sodium formate under CO$_2$ gas-saturated and quiescent conditions at 25 °C. $v = 10$ mV s$^{-1}$. The dotted line represents the CV in the absence of substrate. The broken line shows the expected residual slope. The inset shows the Nernst analysis of the sigmoidal catalytic curve (see text). The normalized voltammetric data are indicated by open circles. The solid curve is the refined curve obtained by non-linear regression analysis based on Eq. (4).

(B) CVs for NADH oxidation and NAD$^+$ reduction at FoDH1-modified Py/AuNP/KB/GCE in 1.0 M phosphate buffer (pH 7.0) containing 50 mM NAD$^+$ and NADH under Ar-saturated and quiescent conditions at 25 °C $v = 10$ mV s$^{-1}$. The dotted line represents the CV in the absence of substrate. The broken line shows the expected residual slope. The inset shows Nernst analysis of the sigmoidal catalytic curve (see text). The normalized voltammetric data are indicated by open circles. The black solid curve is the refined data obtained by non-linear regression analysis based on Eq. (4).

(C) Dependence of the HCOO$^-$ oxidation current density at $-0.3$ V on the common logarithm of the concentration of imidazole ($c_{im}$) with error bars evaluated by Student’s t-distribution at a 90% confidence level.
inset of Fig. 1B shows the fitting results. The refined data are \( n_{\text{app}} = 0.93 \pm 0.02 \) and \( E_{\text{red}}^{\circ} = -0.591 \pm 0.001 \) V at pH 8.0. The electrochemically active and communicating site can be reasonably assigned to an iron-sulfur cluster (FeS\(_a\)). The refined \( n_{\text{app}} \) value is close to unity as the theoretical value for FeS\(_a\). This indicates that the heterogeneous electron transfer between the FeS\(_a\) site and Py/AuNP/KB/GCE is reversible; a Nernstian response is observed for the proteins oriented suitably for the DET reaction, and the intra-molecular electron transfer in FoDH1 from HCOO\(^-\) to the FeS\(_a\) site via the W\(_a\) site is sufficiently fast. The latter electron transfer is thermodynamically downhill at pH 8.0 because the formal redox potential of the CO\(_2\)/HCOO\(^-\) couple (\( E_{\text{CO}_2}^{\circ} = -0.7 \) V [42]) is more negative than \( E_{\text{red}}^{\circ} \).

Considering the above discussion, we can expect an almost reversible catalytic response of the CO\(_2\)/HCOO\(^-\) redox couple at \( E_{\text{CO}_2}^{\circ} \approx E_{\text{red}}^{\circ} \) with FoDH1-adsorbed Py/AuNP/KB/GCE. The solid line in Fig. 2A shows the CV at FoDH1-adsorbed Py/AuNP/KB/GCE in the presence of CO\(_2\) (in practice, hydrogen carbonate) and HCOO\(^-\) at pH 6.6, where \( E_{\text{CO}_2}^{\circ} = -0.61 \) V. These catalytic waves are not observed in the absence of HCOO\(^-\) and CO\(_2\) (Fig. 2A, dotted line) and are assigned to HCOO\(^-\) oxidation and CO\(_2\) reduction catalyzed by FoDH1 adsorbed on Py/AuNP/KB/GCE. The zero-current potential \( (E_j - \eta) \) is practically identical to \( E_{\text{CO}_2} \) as defined by the Nernst equation:

\[
E_{\text{CO}_2} = E_{\text{CO}_2}^{\circ} + \frac{RT}{2F} \ln \frac{p_{\text{CO}_2}}{c_{\text{HCOO}^-}}
\]  

(3)

where \( c_{\text{HCOO}^-} \) is the concentration of HCOO\(^-\) and \( p_{\text{CO}_2} \) is the pressure of CO\(_2\). The current density is approximately three times higher than that recorded at FoDH1-adsorbed KB/GCE [39]. The sigmoidal part (obtained by subtracting the expected residual slope, indicated by a broken line in Fig. 2A) was analyzed using Eqs. (4) and (2):

\[
\frac{j_s(E) - j_{\text{lim,red}}}{j_{\text{lim,ox}} - j_{\text{lim,red}}} = \frac{\eta}{1 + \eta}
\]

(4)

where \( j_{\text{lim,red}} \) is the steady-state limiting reduction current density. The inset of Fig. 2A shows the fitting results. The refined data are \( n_{\text{app}} = 0.56 \pm 0.02 \) and \( E_{\text{red}}^{\circ} = -0.587 \pm 0.004 \) at pH 6.6. The value of \( E_{\text{red}}^{\circ} \) is very close to that obtained at pH 8.0. This indicates that the formal potential of FeS\(_a\) is independent of pH in this pH region. The \( n_{\text{app}} \) value is slightly smaller than unity. The intra-molecular electron transfer from the FeS\(_a\) site to CO\(_2\) via the W\(_a\) site appears to be somewhat slow due to the slightly uphill electron transfer at pH 6.6 (\( E_{\text{red}}^{\circ} = -0.59 \) V > \( E_{\text{CO}_2}^{\circ} = -0.61 \) V).
Figure 2B (solid curve) shows the CV at FoDH1-adsorbed Py/AuNP/KB/GCE in the presence of NAD\(^+\) and NADH at pH 7.0, where the formal redox potential of the NAD\(^+\)/NADH redox couple \(E^{\circ}_{\text{NAD}}\) is \(-0.52\) V [43]. These catalytic waves are not observed in the absence of NAD\(^+\) and NADH (Fig. 2B, dotted line); they are assigned to NADH oxidation and NAD\(^+\) reduction catalyzed by FoDH1 adsorbed on Py/AuNP/KB/GCE. The \(E_j\) value is practically identical to \(E^{\circ}_{\text{NAD}}\) (note that isoconcentrations of NAD\(^+\) and NADH were used in these experiments). The sigmoidal curve (obtained by subtracting the expected residual slope, indicated by a broken line in Fig. 2B) was also analyzed using Eqs. (4) and (2). The inset of Fig. 2B shows the fitting results. The refined data are \(n_{\text{app}} = 0.90 \pm 0.05\) and \(E^{\circ}_{\text{E}} = -0.596 \pm 0.003\) V. The value of \(E^{\circ}_{\text{E}}\) is very close to that obtained from the analysis of the catalytic waves of HCOO\(^-\) oxidation and CO\(_2\) reduction. This indicates that the FeS\(_a\) site in DET-type bioelectrocatalysis for the NAD\(^+\)/NADH redox couple is identical to that in the DET reaction for the CO\(_2\)/HCOO\(^-\) redox couple. The \(j_{\text{s,lim,ox}}\) value is much smaller than \(|j_{\text{s,lim,ox}}|\). This is because the intra-molecular electron transfer from NADH to the FeS\(_a\) site via the FMN catalytic site at pH 7.0 is uphill (\(E^{\circ}_{\text{NAD}} = -0.52\) V > \(E^{\circ}_{\text{E}} = -0.59\) V).

3.3. Interaction between FoDH1 and Py/AuNP/KB/GCEs

All the electrochemical results indicate that Py/AuNP/KB/GCE is a suitable platform for four-way DET-type bioelectrocatalysis of FoDH1. We also investigated the interaction between FoDH1 and the pyridine moiety on the electrode based on our hypothesis that the pyridine moiety can attractively interact with the FeS\(_a\) site in FoDH1, considering a report that a pyridine-terminal unit can coordinate to the iron sulfur unit of (E)-1-hydroxy-2-methylbut-2-enyl 4-diphosphate reductase, a [FeS\(_4\)] cluster-containing enzyme [44]. To test our hypothesis, we examined the effects of imidazole addition on the DET-type bioelectrocatalytic current of HCOO\(^-\) oxidation. Imidazole may specifically disrupt the interaction between the FeS\(_a\) site and the pyridine moiety. Figure 2C shows the amperometric response upon addition of imidazole. When imidazole was added, the HCOO\(^-\) oxidation current density decreased in a concentration-dependent manner. This result supports our hypothesis.

4. Conclusions

We have developed a 4-mercaptopypyridine-treated AuNP-embedded mesoporous carbon electrode as a new platform for DET-type bioelectrocatalysis of FoDH1. AuNPs 5 nm in diameter were embedded on KB/GCE. Microscopic measurements indicated that the AuNPs were uniformly dispersed on the KB surfaces without aggregation. We succeeded in enhancing the activity of DET-
type bioelectrocatalysis of FoDH1 adsorbed on Py/AuNP/KB/GCE. Electrochemical measurements showed that the heterogeneous electron transfer between the FeS₆ site of FoDH1 and the surface of Py/AuNP/KB/GCE is electrochemically reversible. Four-way DET-type bioelectrocatalysis was realized for the CO₂/HCOO⁻ and NAD⁺/NADH redox couples. The reaction is controlled by pH and the electrode potential. The electrochemically active site is reasonably assigned to an ion-sulfur cluster, which has a formal potential of \(-0.591 \pm 0.005\) V that is independent of pH. When an uphill process is involved in the intramolecular electron transfer, the overall reversibility and limiting current density decrease. The pyridine moiety on the mesoporous electrode plays important roles in enhancing the heterogeneous electron transfer and in increasing the probability of suitable enzyme orientation for DET reactions on the electrode. This work also suggests that a variety of redox enzymes can function as DET-type bioelectrocatalysts on suitably tuned and tailored electrode surfaces. Because these DET-type bioelectrocatalytic signals reflect several physicochemical characteristics of the enzyme, these electrochemical methods will open a new avenue for the characterization of redox enzymes.

5. References

Chapter 1, 1-2

Chapter 1, 1-2


Interconversion between formate and hydrogen carbonate by tungsten-containing formate dehydrogenase-catalyzed mediated bioelectrocatalysis

Abstract
We have focused on the catalytic properties of tungsten-containing formate dehydrogenase (FoDH1) from Methylobacterium extorquens AM1 to construct a bioelectrochemical interconversion system between formate (HCOO\(^-\)) and hydrogen carbonate (HCO\(_3^-\)). FoDH1 catalyzes both of the HCOO\(^-\) oxidation and the HCO\(_3^-\) reduction with several artificial dyes. The bi-molecular reaction rate constants between FoDH1 and the artificial electron acceptors and NAD\(^+\) (as the natural electron acceptor) show the property called a linear free energy relationship (LFER), indicating that FoDH1 would have no specificity to NAD\(^+\). Similar LFER is also observed for the catalytic reduction of HCO\(_3^-\). The reversible reaction between HCOO\(^-\) and HCO\(_3^-\) through FoDH1 has been realized on cyclic voltammetry by using methyl viologen (MV) as a mediator and by adjusting pH from the thermodynamic viewpoint. Potentiometric measurements have revealed that the three redox couples, MV\(^{2+}/\)MV\(^{+}\), HCOO\(^-\)/HCO\(_3^-\), FoDH1 (ox/red), reach an equilibrium in the bulk solution when the two-way bioelectrocatalysis proceeds in the presence of FoDH1 and MV. The steady-state voltammograms with two-way bioelectrocatalytic properties are interpreted on a simple model by considering the solution equilibrium.

1. Introduction
In recent years, the technology of capturing and storing renewable energy has been extensively discussed and investigated. The reduction of carbon dioxide to generate reduced carbon compounds for use as fuels and chemical feedstocks is an essential requirement for carbon-based sustainable energy economy (Appel et al., 2013). Interconversion system of formate/carbon dioxide (HCOO\(^-\)/CO\(_2\)) is one of the answers for the purpose. Furthermore, this system has another merit of CO\(_2\) fixation, since CO\(_2\) is known to a major cause of the present global warming (Lashof and Ahuja, 1990). CO\(_2\) fixation helps not only to produce renewable energy and to develop new carbon cycle but also to decrease the atmospheric CO\(_2\) level (Jacobson, 2009). Formate is the first stable intermediate during the reduction of CO\(_2\) to methanol or methane and is increasingly recognized as a new energy source (Rice et al., 2002; Ha et al., 2006). In addition, it can easily be handled, stored, and transported. However, when CO\(_2\) is reduced and formate is oxidized directly on electrodes, a variety of products are generated and quite high overpotential is required (Adić et al., 1980; Hori, 2008). The non-catalyzed thermal decomposition of formate is dominated by the reaction channels, the decarboxylation/dehydrogenation yielding carbon dioxide and hydrogen. For the desired pathway a
higher activation energy is necessary (Enthaler et al., 2010). Catalysts developed so far to overcome this problem are inefficient and expensive (Ruschig et al., 1976; Haines et al., 1994; Hull et al., 2012; Costentin et al., 2012; Kang et al., 2012, 2013; Huff and Sanford, 2013; Smieja et al., 2013; Sampson et al., 2014). One of the most promising strategies for solving these issues is the utilization of enzymes as catalysts. Enzymes have novel properties of substrate specificities and high catalytic efficiencies, allowing them to function in a specific biological reaction under mild conditions, such as room temperature, atmospheric pressure and neutral pH.

Formate dehydrogenase (FoDH) is the key enzyme in the energy conversion reactions of methylotrophic aerobic bacteria, fungi, and plants. The enzyme, in general, catalyzes the oxidation of formate to CO$_2$. However, certain FoDHs have been reported to act as CO$_2$ reductases (Andreesen et al., 1974; Yamamoto et al., 1983; Liu and Mortenson, 1984; Alissandratos et al., 2013; Choe et al., 2014). It is now established that some redox enzymes are able to catalyze reactions reversibly (Armstrong and Hirst, 2011). For example, DMSO-reductase (Hagedoorn et al., 1998; Stewart et al., 2000), CO dehydrogenase (Parkin et al., 2007), fumarate:menaquinone oxidoreductase, succinate:quinone reductase (Léger et al., 2001) and some hydrogenases (Vincent et al., 2007). A great variability is found in bacterial FoDHs and they can be divided into two major classes based on their metal content/structure and consequent catalytic strategies (Maia et al., 2015). The metal-independent FoDH class comprises NAD-dependent FoDHs in the category of the D-specific dehydrogenases of the 2-oxyacid family (Kato et al., 1990; Vinals et al., 1993; Popov and Lamzin, 1994). These enzymes are found in aerobic bacteria, yeast, fungi and plants. Because these enzymes have no redox cofactors or metal ions, the formate oxidation to CO$_2$ has been suggested to involve the direct hydride transfer from formate to NAD$^+$. The metal-containing FoDH class comprises only prokaryotic FoDHs in the category of the molybdenum and tungsten-containing enzyme families. This class of FoDHs is composed of complex subunits with different redox cofactors, and the active site harbors one molybdenum or tungsten atom that catalyzes the proton/electrons transfer in their active site, at which the formate oxidation takes place. Accordingly, the metal-containing FoDH class can be sub-divided as molybdenum-containing FoDH and tungsten-containing FoDH.

Notably, some FoDHs in the metal-containing FoDH class also comprises NAD-linked FoDH, which contains FMN to link with NAD$^+$. These enzymes utilize NAD$^+$ only as the electron acceptor in the biological system. These enzymes are suitable for electrochemistry because some of them can transfer electrons to electrode or artificial redox partners (mediators) (Parkinson and Weaver, 1984; Kuwabata et al., 1994; Reda et al., 2008; Bassegoda et al., 2014). Mediators enable the enzymatic reaction to couple with an electrode reaction by shuttling electrons between enzymes and electrodes. This reaction is called mediated electron transfer (MET)-type bioelectrocatalysis. MET-type reaction has been recognized as a key system for developing novel biosensors, bioreactors and biofuel cells, because a variety of oxidoreductase reactions can be utilized for these applications (Kano
and Ikeda, 2000).

Here, we have focused on tungsten-containing formate dehydrogenases (FoDH1; EC 1.2.1.2) from *Methylobacterium extorquens* AM1. This enzyme is one of the NAD-linked formate dehydrogenases from this methylotroph (Chistoserdova et al., 2007). FoDH1 is heterodimer of two identical subunits each comprising two domains (a coenzyme binding domain and a substrate binding domain) and catalyzes the oxidation of formate to CO$_2$ in coupled reduction of NAD$^+$ to NADH (Laukel et al., 2003). It is difficult to use the NAD$^+$/NADH couple as a mediator in bioelectrocatalytic system, because the electrochemical reaction of the NAD$^+$/NADH couple on electrodes requires very high overpotentials. We must find other mediators such as quinines to couple the enzyme reaction with electrode reactions. FoDH1 uses ferricyanide and several oxidized dyes as electron acceptors in place of NAD$^+$ (Laukel et al., 2003). Therefore, FoDH1 has possibility that it shows the CO$_2$ reduction activity with some suitable reduced dyes as electron donors as like as another FoDHs and can be utilized to construct a bioelectrochemical interconversion system of formate/CO$_2$. In this paper, we have demonstrated that FoDH1 reacts with several artificial mediators as electron donors for the reduction of CO$_2$ to formate as well as acceptors for the oxidation of formate to CO$_2$, and have evaluated the bi-molecular reaction rate constants between FoDH1 and the mediators. Furthermore, we have constructed a bioelectrochemical interconversion system between formate and CO$_2$ using FoDH1 and methyl viologen. The kinetics has been interpreted from the thermodynamic point of view and effects of the reversible property of FoDH1 on the catalytic current response have been detailed. Based on the thermodynamic and kinetic aspects, a strategy to get two-way bioelectrocatalytic system with one mediator has been presented.

2. Experimental

2.1. Materials

Sodium chloride, ammonium sulfate, potassium dihydrogenphosphate, sodium formate, sodium carbonate, sodium molybdate dihydrate, 1-methoxy-5-methylphenazinium methyl sulfate (PMS), 2,6-dichlorophenolindophenol sodium salt hydrate (DCIP), 1,2-naphthoquinone (BNQ), 1,4-naphthoquinone (ANQ), 2-methyl-1,4-naphthoquinone (VK$_3$), anthraquinone-2-sulfonic acid (AQ2S), alizarin red S (ARS) and 1,4-benzoquinone (BQ) were purchased from Wako Pure Chemical (Japan). Benzyl viologen (BV) and sodium tungstate dihydrate were obtained from Nacalai Tesque (Japan). Methyl viologen dichloride (MV), 9,10-phenanthrenequinone (PQ), 2,5-dichloro-1,4-benzoquinone (25DCBQ) and anthraquinone-2,7-disulfonic acid (AQ27DS) were obtained from Tokyo Chemical Industry (Japan). Hipolypepton was sourced from Nihon Seiyaku (Japan). Yeast extract and NAD$^+$ were sourced from Oriental Yeast (Japan). 2,3-Dimethoxy-5-methyl-1,4-benzoquinone (Q$_0$) was
obtained from Sigma-Aldrich Co. (USA). All chemicals were of analytical grade and used as received. The doubly distilled water used for sample and buffer preparation was purified with a Milli-Q water.

2.2. Purification of FoDH1

*Methylobacterium extorquens* AM1 (NCIMB 9133) was purchased from NCIMB (Aberdeen, Scotland, UK). The cells were grown at 28 °C in modified Luria broth, which consisted of 1% hipolypepton, 1% yeast extract, 0.5% sodium chloride, 1 μM sodium tungstate and 0.5 μM sodium molybate. The cells were cultivated in 500-mL Erlenmeyer flasks filled with 150 mL medium. (Warning: When the cells were grown in 10-L glass fermenters containing 6 L medium, the specific activity of FoDH1 was very low (28 U mg⁻¹). Therefore, the cultures were harvested in the method described above.) The cells were collected, suspended with 20 mM potassium phosphate buffer (KPB) pH 6.0 and then disrupted two times with a French pressure cell (Otake Works, Japan) at 100 MPa. Centrifugation was performed at 100,000 × g for 1 h at 4 °C to remove cell debris. The supernatant solution was loaded on a Toyopearl DEAE-650M column (Tosoh Corporation, Japan) equilibrated with 20 mM KPB pH 6.0. FoDH1 was eluted with linear gradient of NaCl from 120 mM to 180 mM in the KPB pH 6.0. The sample was collected and applied to a Toyopearl Butyl-650M column (Tosoh Corporation, Japan) equilibrated with the KPB containing 20% (w/w) ammonium sulfate. The elution of FoDH1 was carried out under a linear gradient of ammonium sulfate from 12% to 8% in the same KPB pH 6.0. All purification steps were performed at 4 °C under aerobic conditions. Protein concentrations were determined with the Pierce® BCA Protein Assay Kit (Thermo Scientific, USA) using bovine serum albumin as a standard. The purities of FoDH1 were judged by Coomassie brilliant blue R-250 staining of SDS-PAGE.

2.3. Spectroscopic measurements

2.3.1. FoDH1 assays

FoDH1 activity assays were done in 1-cm light-path cuvettes with 0.1 M KPB pH 7.0. The 1-mL assay mixture contained 30 mM formate, 0.2 mM DCIP and 0.05 mM PMS. Reactions were started by the addition of the FoDH1, and the decrease in the absorbance at 600 nm due to the reduction of DCIP was measured using a Shimadzu UV-2550 UV-VIS Spectrophotometer (Japan). One unit of FoDH1 activity was defined as the amount of FoDH1 that catalyzes the reduction of 1 μmol of DCIP per min. The extinction coefficient for DCIP at 600 nm was taken as 20.6 mM⁻¹ cm⁻¹ at pH 7.0 (Moscow, 1985). The specific activity of the enzyme purified here was 330 U mg⁻¹.
2.3.2. The kinetics parameter of FoDH1 for NAD$^+$

The kinetic parameter of FoDH1 for NAD$^+$ was determined in 0.1 M KPB pH 7.0 at 30 ± 2 °C. The reaction rates were determined by monitoring the production of NADH at 340 nm in 1-cm light-path cuvettes by a using molar extinction coefficient of 6.22 mM$^{-1}$ cm$^{-1}$ (Hatrongjit and Packdibamrung, 2010).

2.4. Electrochemical measurements

All Electrochemical measurements were carried out with an ALS 611s voltammetric analyzer in 0.1 M KPB at various pH at 30 ± 2 °C under a complete argon atmosphere. The working electrode was a glassy carbon electrode (GCE, 3mm in diameter, BAS). The GCE was polished with 0.05-μm alumina powder, sonicated to remove it and washed with distilled water. The reference and counter electrodes were a handmade Ag|AgCl|sat.KCl electrode and a Pt-wire, respectively. All of the potentials are referred to the reference electrode in this paper. In all of the bioelectrocatalytic experiments, the enzyme and the mediators were used in soluble (un-immobilized) forms.
3. Result and discussion

3.1. Mediated bioelectrocatalysis of formate oxidation

FoDH1 did not give a clear signal of direct electron transfer-type bioelectrocatalysis in the presence of formate (HCOO\(^-\)) at GCEs, as shown in Fig. 1. However, in the presence of PQ as a mediator, a large catalytic oxidation wave with sigmoidal and steady-state characteristics was observed in KPB (pH 7.0) containing 50 mM HCOO\(^-\), as shown in Fig. 2A (solid line). The half-wave potential (−0.18 V) is in a good agreement with the formal potential of PQ (\(E^{\circ'}_{\text{PQ}} = -0.18\) V at pH 7.0, which was evaluated as a mid-point potential in non-turnover voltammetric wave of PQ, given in Fig. 2A, broken line). The sigmoidal wave is a typical mediated bioelectrocatalysis of the HCOO\(^-\) oxidation. Similar bioelectrocatalytic oxidation wave was observed, when MV\(^+/MV^{**}\) with \(E^{\circ'}_{\text{MV}} = -0.63\) V was used as a mediator (Fig. 2B), but the steady-state limiting current was much smaller than that observed in the presence of PQ.

We examined the relation between the formal potential of mediator (\(E^{\circ'}_{\text{M}}\)) and the biomolecular reaction rate reaction constants between FoDH1 and mediators for the HCOO\(^-\) oxidation (\(k_{\text{ox}} = k_{\text{cat,ox}}/K_{M,\text{ox}}\), \(k_{\text{cat,ox}}\) and \(K_{M,\text{ox}}\) being the catalytic constant for the HCOO\(^-\) oxidation and the Michaelis constant for the oxidized form of a mediator, respectively). Under the assumptions that the FoDH1-catalyzed HCOO\(^-\) oxidation follows an ordinary ping-pong bi-bi mechanism, the steady-state kinetics of the HCOO\(^-\) oxidation reaction by FoDH1 is expressed by:

\[
v_{E,\text{ox}} = \frac{n_{\text{HCOO}}}{n_{M}} k_{\text{cat,ox}} \frac{c_{M,\text{ox}}}{1 + \frac{K_{M,\text{ox}}}{c_{\text{HCOO}}}} + \frac{c_{\text{HCOO}}}{c_{M,\text{ox}}}
\]

(1)

Figure 1.
Cyclic voltammograms of FoDH1 in KPB (pH7.0) at \(v = 0.02\) V s\(^{-1}\) and at GC electrode. Dash line : background current in the absence of enzyme, solid line : in the presence of FoDH1 and 50 mM formate.
When the HCOO$^-$ concentration ($c_{\text{HCOO}^-}$) is much larger that the Michaelis constant for HCOO$^-$ ($K_{\text{HCOO}^-} = 1.6 \text{ mM}$ (Laukel et al., 2003)), the steady-state enzymatic reduction rate of the mediator ($v_{\text{M,red}}$) is given by:

$$v_{\text{M,red}} = \frac{n_{\text{HCOO}^-} k_{\text{cat,ox}} c_E}{n_M} \frac{c_{\text{M,ox}}}{1 + \frac{K_{\text{M,ox}}}{c_{\text{M,ox}}}}$$  \hspace{1cm} (2)

where $n_{\text{HCOO}^-} (= 2)$ and $n_M$ are the numbers of electrons of HCOO$^-$ and mediator, respectively, and $c_E$ and $c_{\text{M,ox}}$ are the total concentrations of FoDH1 and the oxidized mediator in the bulk solution, respectively. The value of $k_{\text{ox}}$ for NAD$^+$ was evaluated from the spectrophotometric

**Figure 2.**

Cyclic voltammograms recorded at a scan rate ($v$) of 0.01 V s$^{-1}$ on a bare GC electrode under a complete argon atmosphere for: (A) 44 μM PQ with HCOO$^-$ in the absence (dash line) and presence (solid line) of FoDH1 in 0.1 M KPB (pH 7.0) (B) 44 μM MV with HCOO$^-$ in the absence (dash line) and presence (solid line) of FoDH1 (pH 7.0) (C) 44 μM MV with HCO$_3^-$ in the absence (dash line) and presence (solid line) of FoDH1 in 0.1 M KPB (pH 6.6).
monitoring of NADH during the FoDH1 reaction with NAD$^+$ as an electron acceptor.

Under bioelectrochemical conditions of $c_{\text{HCOO}} >> K_{\text{HCOO}}$, the concentration polarization of HCOO$^-$ becomes negligible near the electrode surface to generate steady-state sigmoidal waves, as shown in Fig. 2. When $c_{M_{\text{red}}} << K_{M_{\text{ox}}}$ ($c_{M_{\text{red}}}$ being the bulk concentration of the reduced mediator), the steady-state catalytic limiting current $i_{\text{lim,ox}}$ of a mediated bioelectrocatalysis is given by (Matsumoto et al., 2002):

$$i_{\text{lim,ox}} = n_M F A_{M_{\text{red}}} \sqrt{(n_{\text{HCOO}} / n_M) D_M k_{\text{ox}} c_E}$$  \hspace{1cm} (3)$$

where $F$, $A$, and $D_M$ are the Faraday constant, the electrode surface area, and the diffusion constant of a mediator, respectively. The diffusion constants were determined by hydrodynamic voltammetry for each mediator in the absence of FoDH1 and its substrate (Table S2). The $k_{\text{ox}}$ values were estimated on the basis of Eq. (3) for the artificial electron acceptors that can work as the mediators.

The logarithmic values of $k_{\text{ox}}$ increase linearly with $E_{\text{ox}}$ of the electron acceptors examined here up to about $–0.2$ V of $E_{\text{ox}}$ and reached a limiting level of $\log(k_{\text{ox}}) \approx 0.85$, as shown in Fig. 3A. As pointed out in a previous paper (Okumura et al., 2006), the maximum value of $k_{\text{ox}}$ is in an order expected for typical diffusion-controlled reactions. In order to construct high performance biofuel cells, a large current density (that is, a rapid mediated bioelectrocatalytic reaction) is required at a potential as negative as possible. Figure 3A indicates that PQ is the most effective mediator for the HCOO$^-$ oxidation to realize a diffusion-controlled reaction with FoDH1.

The linear dependence is called a linear free energy relationship (LFER), which should be observed for reactions with no strong specific interaction, and is given by the following equation for this case.

$$\log \left( \frac{k_{\text{ox},j}}{k_{\text{ox},i}} \right) = \frac{\beta_{\text{ox}} n_{\text{ox}}'}{2.303 RT} \Delta E_{M_{\text{ox}},M_{\text{ox}}}'$$ 

\hspace{1cm} (4)$$

where $\beta_{\text{ox}}$ is a proportional constant in an LFER ($0 < \beta_{\text{ox}} < 1$), $n_{\text{ox}}'$ is the number of electrons in the rate determining step, $R$ is the gas constant, $T$ is the absolute temperature, and $\Delta E_{M_{\text{ox}},M_{\text{ox}}}'$ is the difference in the formal redox potential between mediator $j$ and mediator $i$ ($E_{M_{\text{ox}},M_{\text{ox}}}'$). $\beta_{\text{ox}}$ is evaluated to be about 0.5 by assuming $n_{\text{ox}}' = 1$ from the linear dependence in the $E_{M_{\text{ox}}}'$ range from $–0.7$ V to $–0.2$ V.

It is noteworthy that the log $k_{\text{ox}}$ value of NAD$^+$ also locates on the linear relationship of the
other artificial electron acceptors. This means that the interaction between NAD$^+$ and FoDH1 is not so specific and NAD$^+$ behaves as like as artificial electron acceptors, although NAD$^+$ is regarded as the natural electron acceptor of FoDH1 in the biological system (Laukel et al., 2003).

3.2. Mediated bioelectrocatalysis of the reduction of hydrogen carbonate reduction

As described in section 3.1, the catalytic current of the HCOO$^-$ oxidation was observed with MV in KPB of pH 7.0, as shown in Fig. 2B, solid line. It is noteworthy that the $E^{°}_{M'}$ of MV

![Diagram]

Figure 3.
Logarithmic values of the bi-molecular rate constants between FoDH1 and various mediators for: (A) the HCOO$^-$ oxidation ($k_{ox}$) and (B) the HCO$^-$ reduction ($k_{red}$) as a function of the formal potential of the mediator at 30 °C and in 0.1 M KPB (pH 7.0). The solid lines in panels A and B are LFER lines with a slope identical given by Eqs. (4) and (6), respectively. The broken line in panel A indicates a limiting level of log($k_{ox}$) = 0.85.
(\(E^{\circ}_{\text{MV}} = -0.63\) V) is only slightly more positive than the formal potential (\(E_{S}^{\circ}\)) of the HCOO\(^{-}\)/HCO\(_3\)\(^{-}\) couple at pH 7.0 (= -0.64 V (Hori, 2008)). The successful observation of the catalytic HCOO\(^{-}\) oxidation with MV at pH 7.0 has inspired expectations that FoDH1 can catalyze the reduction of hydrogen carbonate (HCO\(_3\)\(^{-}\)) with MV\(^{**}\) as an electron donor under slightly acidic conditions. Because \(E_{S}^{\circ}\) of the HCOO\(^{-}\)/HCO\(_3\)\(^{-}\) couple shifts to positive direction with a decrease in pH and \(E^{\circ}_{\text{MV}}\) is independent of pH, the electron transfer from MV\(^{**}\) to HCO\(_3\)\(^{-}\) becomes downhill under slightly acidic conditions. In order to examine the HCO\(_3\)\(^{-}\) reduction, cyclic voltammetry was carried out at pH 6.6 in the presence of HCO\(_3\)\(^{-}\) (50 mM) and MV. When FoDH1 was added into the measurement solution, a sigmoidal and steady-state cathodic wave was observed, as shown in Fig. 2C, solid line. The wave is MV-mediated and FoDH1-catalyzed reduction of HCO\(_3\)\(^{-}\). We also attempted to observe the HCO\(_3\)\(^{-}\) reduction current with some other mediators as electron donors and evaluated the bi-molecular reaction rate reaction constant between FoDH1 and mediators for the HCO\(_3\)\(^{-}\) reduction and Michaelis constant for the reduced form of a mediator, respectively) by:

\[
i_{\text{sm,red}} = -n_{M}FAc_{\text{Mox}} \frac{(n_{\text{HCO}_{3}^{-}}/n_{M})D_{M}k_{\text{red}}^{e}c}{E^{\circ}_{\text{M,red}}}
\]

(5)

The physical meanings of the parameters are similar to those of Eq. (3) but are related to the reduction of HCO\(_3\)\(^{-}\) instead of the oxidation of HCOO\(^{-}\). The \(k_{\text{red}}\) values also appear to follow an LFER against \(E^{\circ}_{\text{M}}\).

\[
\log \left( \frac{k_{\text{red,}j}}{k_{\text{red,i}}} \right) = \frac{\beta_{\text{red}}n'_{\text{red}}F}{2.303RT} \Delta E_{\text{M,}j/i}^{\circ}
\]

(6)

The proportional constant \(\beta_{\text{red}}\) (0 < \(\beta_{\text{red}}\) < 1) was evaluated to be about 0.5 by assuming \(n'_{\text{red}} = 1\).

3.3. Effects of pH on the bi-molecular reaction rate constant between FoDH1 and MV

As described in section 3.2, \(E_{S}^{\circ}\) of the HCOO\(^{-}\)/HCO\(_3\)\(^{-}\) couple is a function of pH, while \(E^{\circ}_{\text{MV}}\) is independent of pH. In addition, the catalytic reactions of both of the HCOO\(^{-}\) oxidation and the HCO\(_3\)\(^{-}\) reduction obey the LFER. Therefore, we can expect pH dependence of the bi-molecular reaction rate constant between FoDH1 and MV for the HCOO\(^{-}\) oxidation and the HCO\(_3\)\(^{-}\) reduction.

By considering the standard formal redox potential of the HCOO\(^{-}\)/HCO\(_3\)\(^{-}\) couple

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(\(E_s^\circ\) (pH 7)) and p\(K_a\) values (Reda et al., 2008; Bassegoda et al., 2014), \(E_s^\circ\) in pH region from 6.8 to 9.0 at 30 °C is given by:

\[ E_s^\circ = -0.224 \text{ V} - 2.303 \frac{m_S RT}{n_S F} \text{pH} = -0.224 \text{ V} - (0.061 \text{ V}) \times \text{pH} \] (7)

where \(m_S\) is the number of protons and \(n_S\) is the number of electrons in the redox reaction of the HCOO\(^{-}\)/HCO\(_3\)^{-} couple. Equation (7) indicates a two-electron two-proton redox reaction. Therefore, by considering the LFER given by Eqs. (4) and (6), the pH dependence of the bi-molecular reaction rate constant between FoDH1 and MV can be written by:

\[ \log \left( \frac{k_{\text{ox},j}}{k_{\text{ox},i}} \right) = \beta_{\text{ox}} n'_{\text{ox}} \Delta \text{pH}_{ji} \] (8)

for the HCOO\(^{-}\) oxidation and

\[ \log \left( \frac{k_{\text{red},j}}{k_{\text{red},i}} \right) = -\beta_{\text{red}} n'_{\text{red}} \Delta \text{pH}_{ji} \] (9)

for the HCO\(_3\)^{-} reduction, respectively, where \(\Delta \text{pH}_{ji}\) is the difference between pH\(_i\) and pH\(_j\).

We measured both of the anodic and cathodic catalytic currents at various pHs and evaluated \(k_{\text{ox}}\) and \(k_{\text{red}}\) values by using Eqs. (3) and (5), respectively. Figure 4 illustrates logarithmic values of the bi-molecular rate constant against pH for (A) the HCOO\(^{-}\) oxidation and (B) the HCO\(_3\)^{-} reduction. The

![Figure 4](image-url)

**Figure 4.**
Logarithmic plots of (A) \(k_{\text{ox}}\) and (B) \(k_{\text{red}}\) against pH. The solid lines in panel A and B are LFER lines with a slope identical with Eq. (8) and Eq. (9), respectively. The error bars were evaluated by the Student t-distribution at 90% confidential level.
linear relationship was observed between the logarithmic rate constant and pH. The straight lines in the panels have a slope expected from Eqs. (8) and (9) with $\beta_{\text{ox}} = 0.5, n'_{\text{ox}} = 1, \beta_{\text{red}} = 0.5, \text{ and } n'_{\text{red}} = 1$. The results also support the LFER.

3.4. Two-way bioelectrocatalysis of $\text{HCOO}^-$ and $\text{HCO}_3^-$

Based on the above results, we can safely conclude that MV can work as a two-way (both anodic and cathodic) mediator when $\Delta G^o \approx 0$ (that is, $E^o_{\text{S}} \approx E^o_{\text{MV}}$) in the presence of both of $\text{HCO}_3^-$ and $\text{HCOO}^-$. Hence, we measured cyclic voltammograms in KPB (pH 7.0) containing MV, $\text{HCO}_3^-$ (50 mM) and $\text{HCOO}^-$ (50 mM). In the absence of FoDH1, non-turnover redox wave of MV was observed (Fig. 5 dash line). After addition of FoDH1, a steady-state sigmoidal-shaped wave appeared with both cathodic and anodic directions (Fig. 5, solid line). A similar two-way reaction has been reported for the catalytic reaction of *Desulfovibrio vulgaris* cells and $\text{H}_2$ (Tatsumi et al., 1999). To examine the effect of pH against the two-way bioelectrocatalytic voltammogram, we recorded cyclic voltammograms at various pHs in solutions with the same composition except the buffer components. Figure 6 shows that the steady-state current-potential curve after being corrected for the background currents at pH 6.8 (◆), pH 7.3 (▲) and pH 8.4 (■). The sigmoidal steady-state current-potential curve is shifted upward with an increase in pH.

We also performed potentiometry at several pHs in the range from 6.8 to 9.0 in the presence of FoDH1 and MV in solutions containing $\text{HCO}_3^-$ (50 mM) and $\text{HCOO}^-$ (50 mM). The potential

![Figure 5](image_url)

**Figure 5.**
Cyclic voltammograms of the $\text{HCOO}^-$ oxidation and the $\text{HCO}_3^-$ reduction by FoDH1 at $v = 0.01$ V and at a bare GC electrode in 0.1 M KPB (pH 7.0) under a complete argon atmosphere. Dash line: 44 $\mu$M MV + FoDH1, solid line: 44 $\mu$M MV + FoDH1 + 50 mM $\text{HCOO}^-$ + 50 mM $\text{HCO}_3^-$. 

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reached stable values within 10 min after the addition of MV to the reaction buffer containing FoDH1 and the substrates. The equilibrated potential obtained \( E_{eq} \) is plotted against pH of the solution in Fig. 7. The straight line of the \( E_{eq} \) vs. pH plot can be expressed by a linear regression analysis as:

\[
E_{eq} = -(0.056 V) \times \text{pH} - 0.233 \text{ V}
\]  

(10)

This regression equation is in good agreement with the theoretical one (Eq. (7)). This implies that the electrode potential is well equilibrated by the HCO\(_3^+\)/HCOO\(^-\) couple in the solution in the presence of FoDH1 and MV, as expressed by:

\[
E_{eq} = E_{MV}^{eq} + \frac{RT}{F} \ln \frac{c_{MV^{2+}}}{c_{MV^{+}}} = E_{S}^{eq} + \frac{RT}{2F} \ln \frac{c_{HCO_3^-}}{c_{HCOO^-}}
\]  

(11)

When \( c_{MV^{+}} << K_{MV^{+}} \) and \( c_{MV^{2+}} << K_{MV^{2+}} \), the overall rate constant of the MV\(^{2+}\) reduction \( (v_{MV, red}) \) in the FoDH1 reaction can be written by

\[
v_{MV, red} = (n_S/n_M) (k_{ox} c_{E_{ox}} c_{MV^{2+}} - k_{red} c_{E_{red}} c_{MV^{+}})
\]  

(12)

where \( c_{E_{ox}} \) and \( c_{E_{red}} \) are the concentration of the fully oxidized and reduced form of the enzyme in the bulk solution. In the steady state, this equation can be solved by the theory of the steady-state catalytic current \( (i_s) \) of MET-type bioelectrocatalysis reaction (Tatsumi et al., 1999) to give an

**Figure 6.**
Background current-corrected steady-state voltammograms of 44 μM MV + FoDH1 + 50 mM HCOO\(^-\) + 50 mM HCO\(_3^+\) at pH 6.8 (◆), pH 7.3 (▲) and pH 8.4 (■). The solid lines are the regression curve obtained on the basis of Eq. (13) with adequate parameters described in the text.
equation:

\[ i_s = n_M F A c_{MV} \sqrt{\left( \frac{n_S}{n_M} \right) D_{MV} \left( k_{ox} c_{E_{ox}} + k_{red} c_{E_{red}} \right)} \left\{ \frac{1}{1 + K} - \frac{1}{1 + \eta} \right\} \]  

(13)

with

\[ \eta = \frac{c_{MV^2-}}{c_{MV^{2+}}} \bigg|_{x=0} = \exp \left[ \frac{F}{RT} \left( E - E_{MV}^{o} \right) \right] \]  

(14)

\[ K = \frac{c_{MV^2-}}{c_{MV^{2+}}} \bigg|_{x=\infty} = \exp \left[ \frac{F}{RT} \left( E_{eq}^{o} - E_{MV}^{o} + \frac{RT}{2F} \ln \frac{c_{HCOO^-}}{c_{HCO_3^-}} \right) \right] = \exp \left[ \frac{F}{RT} \left( E_{eq}^{o} - E_{MV}^{o} \right) \right] \]  

(15)

Note that, in a strictly sense, the term “HCO$_3$\(^{-}\)” should encompass dissolved CO$_2$, H$_2$CO$_3$, HCO$_3$\(^{-}\) and CO$_3$\(^{2-}\) equilibrated in aqueous solution, and the term “HCOO\(^{-}\)” should encompass HCOOH and HCOO$^-$(Palmer and Eldik, 1983).

Equation (13) was fitted to the experimental steady-state voltammograms with two adjustable parameters \((k_{ox} c_{E_{ox}} + k_{red} c_{E_{red}})\) and \(K\) using a non-linear regression analysis program (Gnuplot$^\text{®}$). The following values were used for the analysis: \(n_M = 1, n_S = 2, F = 96500 \text{ C mol}^{-1}, A = 0.0707 \text{ cm}^2, D_{MV} = 1.36 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}, R = 8.31 \text{ J mol}^{-1} \text{ K}^{-1}, \) and \(T = 303 \text{ K}\). As shown in Fig. 6, the

Figure 7.

pH dependence of \((\bigodot) E_{eq}^{o}\) evaluated by potentiometry, \((\bigcirc) E_{eq}^{o}\) calculated from the \(K\) values evaluated based on Eq. (15), \((\bullet) E_{E=0}\) evaluated from the steady-state voltammograms (Fig. 6), and \((\times) E_{MV}^{o}\) calculated based on Eq. (16). The error bars for \(E_{eq}^{o}\) were evaluated by the Student \(t\)-distribution at 90% confidential level. The solid line is the theoretical line for the standard redox potential of the HCOO$^-$/HCO$_3$\(^{-}\) couple (Eq. (7)). The dash line is a best fit of the data \((\times)\) (Eq. (17)).
experimental data are well reproduced by Eq. (13). The refined parameters are: 

\[ (k_{\text{ox}}c_{E_{\text{red}}} + k_{\text{red}}c_{E_{\text{ox}}}) \]

\[ = (0.36 \pm 0.02) \text{ s}^{-1} \text{ and } K = 2.0 \pm 0.1 \text{ at pH 6.8, } (k_{\text{ox}}c_{E_{\text{red}}} + k_{\text{red}}c_{E_{\text{ox}}}) = (0.30 \pm 0.01) \text{ s}^{-1} \text{ and } K = 1.20 \]

\[ \pm 0.03 \text{ at pH 7.4, and } (k_{\text{ox}}c_{E_{\text{red}}} + k_{\text{red}}c_{E_{\text{ox}}}) = (0.25 \pm 0.02) \text{ s}^{-1} \text{ and } K = 0.18 \pm 0.01 \text{ at pH 8.6.} \]

The error bars are given as the asymptotic standard error obtained by the non-linear regression analysis of the voltammograms. \((N = 3)\) The values of \(E_{\text{eq}}\) were calculated from the evaluated \(K\) values with Eq. \((15)\) and are plotted in Fig. 7. The calculated \(E_{\text{eq}}\) values locate on the line of the potentiometric data \((\text{Eq. 10})\). In addition, Eq. \((13)\) predicts that the steady-state current becomes zero at \(K = \eta\), at which the system becomes a completely equilibrated state. The zero current potential \((E_{i=0})\) on the steady-state voltammograms \((\text{Fig. 6})\) are also plotted in Fig. 7. The good agreement is obtained between \(E_{i=0}\) and \(E_{\text{eq}}\). All these results support the validity of the simple model for a steady-state voltammograms described by Eq. \((13)\).

Since it has been evidenced here that the two redox couples \(\text{MV}^{2+}/\text{MV}^{+}\) and \(\text{HCOO}^{-}/\text{HCO}_{3}^{-}\) are in equilibrium in solution, we can expect that FoDH1 is also in a redox equilibrium in the bulk solution. Therefore, once we can get values of \(c_{E_{\text{red}}} / c_{E_{\text{ox}}}\), the formal potential of FoDH1 \((E_{E}^{\circ})\) can be evaluated by:

\[ E_{\text{eq}} = E_{E}^{\circ} + \frac{RT}{F} \ln \frac{c_{E_{\text{ox}}}}{c_{E_{\text{red}}}} \]  \quad (16)

The ratio of \(c_{E_{\text{ox}}} / c_{E_{\text{ox}}}\) was calculated here from the \((k_{\text{ox}}c_{E_{\text{red}}} + k_{\text{red}}c_{E_{\text{ox}}})\) values evaluated in the non-linear regression analysis and the \(k_{\text{ox}}\) and \(k_{\text{red}}\) values interpolated on the lines in Fig. 4. The evaluated \(E_{E}^{\circ}\) values are plotted in Fig. 7. The broken line is given by a linear regression analysis as:

\[ E_{E}^{\circ} = -(0.061 \text{ V}) \times \text{pH} - 0.183 \text{ V} \]  \quad (17)

The value of \(-0.61 \text{ V}\) corresponding to the standard formal potential of the catalytic redox center of FoDH1 \((E_{E}^{\circ} (\text{pH} 7.0))\) and the slope indicates a 2-electron 2-proton coupled electron transfer.
4. Conclusions

We have demonstrated that FoDH1 can utilize several artificial electron acceptors for the HCOO\(^-\) oxidation. Although the characteristics of the artificial electron acceptors are entirely different from that of the native electron acceptor, NAD\(^+\), the reaction kinetics between FoDH1 and various electron acceptors including NAD\(^+\) obey an LFER. This indicates that the interaction between FoDH1 and NAD\(^+\) is not so specific. Therefore, the electron acceptors except NAD\(^+\) can be utilized as mediators in bioelectrocatalysis of FoDH1. The present result indicates that PQ is the most effective mediator for the HCOO\(^-\) oxidation to reach diffusion controlled condition between FoDH1 and PQ.

On the other hand, we have also reported that FoDH1 can catalyze the HCO\(_3\)\(^-\) reduction with MV\(^{2+}\) and some other reduced dyes. When MV is utilized as a mediator, a two-way bioelectrocatalysis without overpotentials has been realized by controlling pH and the concentration of the substrates. The three redox couples, MV\(^{2+}/MV^{+}\), HCOO\(^-\)/HCO\(_3\)\(^-\), FoDH1 (ox/red), reach an equilibrium in the bulk solution when the two-way bioelectrocatalysis proceeds in the presence of FoDH1 and MV. The voltammogram of the two-way bioelectrocatalysis has been well interpreted on a simple model of MET-type bioelectrocatalysis. The present results are very useful to construct an effective bioelectrochemical reaction for the CO\(_2\) reduction and formate/oxygen biofuel cells as effective energy conversion systems.

5. References


Chapter 2

Development of Bioelectrocatalytic Interconversion System between Carbon Dioxide and Formate

2-1. Bioelectrocatalytic formate oxidation and carbon dioxide reduction at high current density and low overpotential with tungsten-containing formate dehydrogenase and mediators

Abstract

We show a great possibility of mediated enzymatic bioelectrocatalysis in the formate oxidation and the carbon dioxide (CO\textsubscript{2}) reduction at high current densities and low overpotentials. Tungsten-containing formate dehydrogenase (FoDH1) from Methylobacterium extorquens AM1 was used as a catalyst and immobilized on a Ketjen Black-modified electrode. For the formate oxidation, a high limiting current density ($j_{\text{lim}}$) of ca. 24 mA cm\textsuperscript{-2} was realized with a half wave potential ($E_{1/2}$) of only 0.12 V more positive than the formal potential of the formate/CO\textsubscript{2} couple ($E_{\text{o}^{\prime}\text{CO}_2}$) at 30 °C in the presence of methyl viologen (MV\textsuperscript{2+}) as a mediator, and $j_{\text{lim}}$ reached ca. 145 mA cm\textsuperscript{-2} at 60 °C. Even when a viologen-functionalized polymer was co-immobilized with FoDH1 on the porous electrode, $j_{\text{lim}}$ of ca. 30 mA cm\textsuperscript{-2} was attained at 60 °C with $E_{1/2} = E_{\text{o}^{\prime}\text{CO}_2} + 0.13$ V. On the other hand, the CO\textsubscript{2} reduction was also realized with $j_{\text{lim}} \approx 15$ mA cm\textsuperscript{-2} and $E_{1/2} = E_{\text{o}^{\prime}\text{CO}_2} - 0.04$ V at pH 6.6 and 60 °C in the presence of MV\textsuperscript{2+}.

1. Introduction

Electro-enzymatic devices have received considerable attention in view of clean technology to produce electricity and useful materials from renewable fuel sources. An interconversion system of the formate/carbon dioxide (HCOO\textsuperscript{-}/CO\textsubscript{2}) couple is one of the promising objects for such devices (strictly speaking, CO\textsubscript{2} exists as hydrogen carbonate (HCO\textsubscript{3}\textsuperscript{-}) around neutral pH, but we may simply use “CO\textsubscript{2}” in this paper). HCOO\textsuperscript{-} is an energy-rich compound and can be used as a fuel of energy conversion systems such as HCOO\textsuperscript{-}/O\textsubscript{2} biofuel cells, which are comparable to H\textsubscript{2}/O\textsubscript{2} biofuel cells in terms of the theoretical standard electromotive force (ca. 1.2 V [1,2,3]). On the other hand, an efficient bioelectrochemical system of the CO\textsubscript{2} reduction may help us to produce energy-rich products or useful organic chemicals [4] and to reduce the atmospheric CO\textsubscript{2} level under mild conditions [5].

Recently, we have electrochemically characterized tungsten-containing formate dehydrogenase from Methylobacterium extorquens AM1 (FoDH1; EC 1.2.1.2) as a heterodimeric
soluble enzyme [6]. It has been shown that FoDH1 produces mediated bioelectrocatalytic currents for both of the HCOO\(^-\) oxidation and the CO\(_2\) reduction. From the viewpoint of the kinetics between the enzyme and mediators, 9,10-phenanthrenequinone (PQ) is the most effective mediator for the HCOO\(^-\) oxidation. From the thermodynamic viewpoint, methyl viologen (MV\(^{2+}\), 1,1'-dimethyl-4,4'-bipyridinium ion) is an useful mediator for the reversible catalytic reaction of FoDH1 because the midpoint potential of the MV\(^{2+}/MV^{+}\) couple \((E_{m,MV})\) is very close to the formal potential of the HCOO\(^-\)/CO\(_2\) couple \((E^{\circ}_{\text{HCOO}^-/\text{CO}_2})\) around neutral pH. FoDH1 is expected to be applied to the construction of efficient electro-enzymatic devices utilizing the interconversion between HCOO\(^-\) and CO\(_2\).

In such bioelectrochemical devices, a large current density \((j)\) should be realized at potentials close to \(E^{\circ}_{\text{HCOO}^-/\text{CO}_2}\). A promising approach to increase \(j\) is to immobilize enzymes and mediators on an electrode surface. For this purpose, Heller’s group has developed osmium polymers to successfully immobilize enzymes and mediators on electrodes for a mediated electron transfer (MET)-type bioelectrocatalytic system [7]. By using this method, Tsujimura et al. recorded 100 mA cm\(^{-2}\) of the limiting current density \((j_{\text{lim}})\) at 25 °C for a glucose oxidation [8]. However, very large overpotentials \((\approx 1\text{ V})\) are required in such bioelectrochemical glucose oxidation systems to get large \(j_{\text{lim}}\) because of a large formal potential difference between the substrate and the enzyme and of a large kinetic hindrance between the enzyme and the Os polymers. Development of a new mediated bioelectrocatalytic system with small overpotentials is needed.

In this paper, we attempt to construct a MET-type bioelectrocatalytic system for the HCOO\(^-\) oxidation and the CO\(_2\) reduction with high \(j_{\text{lim}}\) and very small overpotentials by using FoDH1. PQ, MV\(^{2+}\), and a viologen-functionalized polymer (VP) were used as mediators. In addition, we focused on Ketjen Black (KB) as an electrode material to effectively immobilize FoDH1. The high \(j_{\text{lim}}\) of ca.145 mA cm\(^{-2}\) was realized at 60 °C without the overpotential in practice for the HCOO\(^-\) oxidation by using free MV\(^{2+}\). Co-immobilization of FoDH1 and mediators was also attempted for practical purpose. The bioelectrochemical CO\(_2\) reduction with high \(j_{\text{lim}}\) was also realized.

2. Experimental

2.1. Chemicals

KB was kindly donated from Lion Co. (Japan). Poly(tetrafluoroethylene) fine powder (PTFE, 6-J) was obtained from DuPont Mitsui Fluorochemicals (Japan). MV\(^{2+}\) dichloride and PQ were obtained from Tokyo Chemical Industry (Japan). Poly(vinylpyrrolidone) (PVP) and poly(ethylene glycol) diglycidyl ether (PEGDGE) were obtained from Sigma-Aldrich Co. (USA). Other chemicals were obtained from Wako Pure Chemical (Japan). FoDH1 was purified according to the literature [6].

60
2.2. Preparation of FoDH1-modified electrodes

KB slurry (KB:PTFE = 8:2 (w/w) in 2-propanol) was applied on a glassy carbon electrode (GCE) for rotating disk voltammetry, and a KB-modified GCE (KB/GCE) was prepared according to the literature [9]. The projective surface area of the GCE was 0.071 cm$^2$. FoDH1 was immobilized on the KB/GCE by immersing the electrode in 40 μL of an FoDH1 solution (50 μM of FoDH1 solution in 100 mM potassium phosphate buffer (KPB) of pH 6) containing 0.5 % (v/v) glutaraldehyde for 24 h at 4 °C. The bioelectrode is called FoDH1/KB/GCE.

2.3. Preparation of FoDH1/PQ co-immobilized KB/GCEs

A PQ-modified KB/GCE (PQ/KB/GCE) was prepared by immersing the KB/GCE in DMSO containing 100 mM of PQ for 12 h at room temperature. After washing the electrode with 100 mM KPB (pH 7.0), FoDH1 was immobilized on the PQ/KB/GCE in a manner similar to that described in Section 2.2. The bioelectrode is called FoDH1/PQ/KB/GCE.

2.4. Preparation of VP and FoDH1/VP co-immobilized KB/GCEs

\(N\)-(4-Bromobutyl)-\(N'\)-methyl-4,4'-bipyridinium dibromide was synthesized according to the literature [10]. VP was prepared by dissolving \(N\)-(4-bromobutyl)-\(N'\)-methyl-4,4'-bipyridinium dibromide (1.1 g, 3 mmol) and PVP (0.5 g, 10 μmol) in 100 mL of DMF, and the solution was stirred for 4 d at 45 °C. The product (VP) was precipitated in diethyl ether and dried. Four μL of 100 mM KPB (pH 7.0) reaction solution containing 90 mg mL$^{-1}$ VP, 20 mg mL$^{-1}$ PEGDGE, and 18 mg mL$^{-1}$ FoDH1 was cast onto the surface of the KB/GCE. The electrode was dried at 4 °C for 3 h. The bioelectrode is called FoDH1/VP/KB/GCE.

2.5. Electrochemical measurements

All electrochemical measurements were carried out in 1.0 M KPB at various pHs and at various temperatures under a complete argon atmosphere on an electrochemical analyzer BAS CV-50W. The working electrodes were rotated with a RDE-1 (BAS, USA). A homemade Ag|AgCl|sat.KCl electrode and a Pt-wire were used as the reference electrode and the counter electrode, respectively. All of the potentials are referred to the reference electrode in this paper.
3. Results and Discussion

3.1 HCOO\(^{-}\) oxidation at FoDH1-modified KB/GCEs

Fig. 1A shows rotating disk cyclic voltammograms (RDVs) at FoDH1/KB/GCEs in the presence of HCOO\(^{-}\) and MV\(^{2+}\). The \(E_{m,MV}\) was \(-0.63\) V. The sigmoidal curves represent the catalytic oxidation of HCOO\(^{-}\), in which FoDH1 and MV\(^{2+}\) work as a catalyst and a mediator, respectively. The catalytic current completely disappeared by HCl-treatment of the bioanode. The current reached the limiting value at potentials more positive than \(-0.45\) V and the half-wave potential \((E_{1/2})\) was \(-0.58\) V, which is only 0.12 V more positive than \(E^{\circ}_{\text{CO2}}\) \((-0.70\) V at pH 8.0 [11]). The \(j_{\text{lim}}\) value increased with the bulk concentration of MV\(^{2+}\) \((c_{MV})\) in a manner of Michaelis Menten-type saturation curve (Fig. 1A, inset). The apparent Michaelis constant against MV\(^{2+}\) was estimated to be \(1.0 \pm 0.2\) mM, the error being an asymptotic standard one in non-linear regression. The \(j_{\text{lim}}\) value was almost independent of the rotation rate \(\omega\) at \(\omega > 600\) rpm (Fig. 1B), indicating that \(j_{\text{lim}}\) is predominantly governed by the enzymatic kinetics. Under the conditions, \(j_{\text{lim}}\) of the HCOO\(^{-}\) oxidation reached \(24 \pm 3\) mA cm\(^{-2}\) at 30 °C and at \(\omega = 1,000\) rpm \((\sqrt{\omega} = 10.23\) s\(^{-1/2}\)).

When the solution temperature was increased up to 60 °C to improve the enzymatic kinetics, \(j_{\text{lim}}\) showed a clear dependence on \(\omega\) and was enhanced to \(145 \pm 6\) mA cm\(^{-2}\) at \(\omega = 6000\) rpm (Fig. 1C). This is the highest \(j_{\text{lim}}\) reported so far for the HCOO\(^{-}\) oxidation at enzymatic bioanodes. The inset in Fig. 1C shows Koutecký-Levich plot based on: [12]

\[
\frac{1}{j_{\text{lim}}} = \frac{1}{j_D} + \frac{1}{j_{\text{cat}}}
\]

(1)

where, \(j_D\) is the Levich-type diffusion-controlled current density and \(j_{\text{cat}}\) is the enzymatic kinetics-controlled current density. The non-linear regression analysis by Gnuplot® with Eq. (1) provided a result that \(j_{\text{cat}} = 294 \pm 52\) mA cm\(^{-2}\) and \(j_D/\omega^{1/2} = 11 \pm 1\) mA s\(^{1/2}\) cm\(^{-2}\). The large value of \(j_{\text{cat}}\) indicates that this FoDH1-based system is very useful for the HCOO\(^{-}\) oxidation.
Since PQ is a better electron acceptor than the natural one (NAD$^+$) from the kinetic
viewpoint [6], we tried to use PQ as a mediator and prepared FoDH1/PQ/KB/GCEs to construct a useful bioelectrocatalytic system for the HCOO⁻ oxidation. PQ adsorbed on a KB/GCE in a manner of Langmuir isotherm (data not shown). Absorbed PQ gave a pair of redox peaks (Fig. 2; b0) at $E_{m} = -0.20$ V. The electrodes produced large anodic currents of the catalytic oxidation of HCOO⁻ (Fig. 2; b1, b2). The $j_{\text{lim}}$ value was almost independent of $\omega$ at 30 °C, indicating the characteristics predominantly controlled by the enzymatic kinetics. The $j_{\text{lim}}$ increased up to ca. 35 mA cm⁻² at 60 °C, however, is much smaller than that expected from the bimolecular rate constant (which is 1,000 times larger than that of MV²⁺ [6]), in spite of a rather large overpotential. Most probably, restricted movement of the adsorbed PQ interferes with the enzymatic reaction between the adsorbed PQ and the immobilized FoDH1.

Although the MV²⁺-system shows very small overpotential, MV²⁺ is very soluble and it is difficult to immobilize MV²⁺ on electrodes. Therefore, we synthesized VP and immobilized it on KB/GCEs. The $E_{m,VP}$ was −0.52 V (Fig. 2; a0) and slightly more positive than $E_{m,MV}$, probably due to the repulsion between the positive charge in the oxidized polymer. In the presence of HCOO⁻, the FoDH1/VP/KB/GCEs produced large anodic $j$ (Fig. 2; a1, a2) comparable with that of FoDH1/PQ/KB/GCEs. The $j_{\text{lim}}$ value reached ca. 30 mA cm⁻² at 60 °C and at $\omega = 1000$ rpm. The $E_{1/2}$ was −0.57 V and only 0.13 V more positive than $E^{\circ'}_{\text{CO}_2}$. These results suggest that the VP-immobilized system can well mediate the electron transfer between the immobilized FoDH1 and the KB/GCE compared with the PQ-adsorbed system. Such a polymer system seems to retain some extent of the

![Figure 2.](image)

RDVs for the HCOO⁻ oxidation at FoDH1/VP/KB/GCEs (curves a1, a2) and at FoDH1/PQ/KB/GCEs (curves b1, b2) in the presence of 500 mM HCOO⁻ at 30 °C (a1, b1) and 60 °C (a2, b2) at pH 8.0. The scan rate was 10 mV s⁻¹ at $\omega = 1,000$ rpm. The dotted lines represent cyclic voltammograms at the FoDH1/VP/KB/GCE (a0) and at the FoDH1/PQ/KB/GCE (b0) in the absence of HCOO⁻.
mobility, which is essential in MET-type bioelectrocatalysis. The flexibility seems to increase at increased temperature, which reduces the electrostatic repulsion of the oxidized polymer, as evidenced by the negative shift in $E_{\text{in,VP}}$ (data not shown).

3.2. $CO_2$ reduction at FoDH1-modified KB/GCEs

With a decrease in pH, $E^{\circ}CO_2$ shifts to the positive potential direction and the electron transfer from $MV^{+•}$ to $CO_2$ becomes downhill. However, the optimum pH of FoDH1 is in the basic range \([13]\) and the enzyme activity decreases with a decrease in pH. Therefore, we examined the $CO_2$ reduction at pH 6.6 by using FoDH1/KB/GCEs. In the presence of $CO_2$ and $MV^{2+}$, FoDH1/KB/GCEs gave steady-state currents of the $CO_2$ reduction (Fig. 3), where $MV^{+•}$ generated from $MV^{2+}$ on the electrode works as an electron donor of the enzyme reaction. The $j_{\text{lim}}$ value was 6 mA cm$^{-2}$ at 30 °C, pH 6.6, and 1,000 rpm. Upon increasing the solution temperature to 60 °C, $j_{\text{lim}}$ was enhanced to 15 mA cm$^{-2}$ at −0.8 V. The $E_{1/2}$ was −0.65 V and is only 0.04 V more negative than $E^{\circ}CO_2$. This is the first report of the enzyme-based bioelectrocatalytic $CO_2$ reduction at such a high $j_{\text{lim}}$.

![Figure 3](image.png)

**Figure 3.**
RDVs for the $CO_2$ reduction at FoDH1/KB/GCEs in the presence of 200 mM $CO_2$ ($HCO_3^-$) and 50 mM $MV^{2+}$ at (a) 30, (b) 40, (c) 50 and (d) 60 °C, and at pH 6.6. The scan rate was 10 mV s$^{-1}$ at $\omega = 1,000$ rpm. The dotted line represents the cyclic voltammogram of the FoDH1/KB/GCE in the presence of 50 mM $MV^{2+}$ under quiet conditions, but in the absence of $CO_2$. 
4. Conclusion

We have demonstrated that FoDH1-immobilized KB electrodes can work as useful bioanodes for the HCOO$^-$ oxidation and useful biocathodes for the CO$_2$ fixation system. FoDH1 immobilized on a KB/GCE surface produces large $j_{\text{lim}}$ with low overpotentials using free MV$^{2+}$ as a mediator for both of the HCOO$^-$ oxidation and the CO$_2$ reduction. VP is also a useful mediator for the HCOO$^-$ oxidation. Such a polymer-based mediator has a great merit in ease of the immobilization with retaining some extent of the mobility required for MET-type reactions. The HCOO$^-$ oxidation system can be applied to the construction of high-powered HCOO$^-$/O$_2$ biofuel cells. In contrast, for the CO$_2$ reduction, it seems to be important to develop other VP with more negative redox potentials for practical use. Introduction of electron-donating substituents to VP is required.

6. References

2-2. High-Power Formate/Dioxygen Biofuel Cell Based on Mediated Electron Transfer Type Bioelectrocatalysis

ABSTRACT

A high-power mediated electron transfer type formate (HCOO\(^-\))/dioxygen (O\(_2\)) biofuel cell is reported herein. The cell utilizes a Ketjen Black modified waterproof carbon cloth as the electrode material. The bioanode comprises tungsten-containing formate dehydrogenase and a viologen-functionalized polymer, whereas the biocathode comprises bilirubin oxidase and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate). In addition, a gas diffusion type system was employed for the biocathode to realize a high-speed O\(_2\) supply. These electrodes exhibited a large current density of 20 mA cm\(^{-2}\) in the quiescent steady state for both HCOO\(^-\) oxidation and O\(_2\) reduction. Finally, these electrodes were coupled to construct an HCOO\(^-\)/O\(_2\) biofuel cell without a separator. The cell exhibited a maximum power density of 12 mW cm\(^{-2}\) at a cell voltage of 0.78 V under quiescent conditions and an open-circuit voltage of 1.2 V. We show the great potential of HCOO\(^-\) for the fuel of biofuel cells.

1. INTRODUCTION

Biofuel cells are energy conversion devices that utilize enzymes as electrocatalysts.\(^1\)\(^-\)\(^8\) On account of the physiological role of enzymes, biofuel cells exhibit unique properties such as high substrate specificity and high catalytic efficiency. The characteristics of the enzyme reactions allow the device to operate under mild and safe conditions (neutral pH, room temperature, and atmospheric pressure). Therefore, biofuel cells have attracted attention for constructing a clean and highly efficient energy conversion system. Bioelectrocatalysis, in which an enzyme reaction is coupled with an electrode reaction, is a key process for biofuel cells.\(^1\)\(^-\)\(^8\) The reaction is classified into two types: mediated electron transfer (MET) and direct electron transfer (DET). In the MET-type reaction system, various organic and inorganic compounds and some redox proteins are used as mediators that shuttle electrons between an electrode and an enzyme. In the DET-type reaction system, the electron transfer occurs directly between an enzyme and an electrode. An enzyme absorbs on an electrode, and electrons tunnel between the electrode and the redox center of the enzyme covered by peptide chains (thick insulators).

Regarding the performance of biofuel cells, the MET-type reaction system is widely utilized in biofuel cells, because the system can be applied to most redox enzymes and the catalytic current is easily improved by increasing the surface concentration of the enzyme and the mediator.\(^9\)

In principle, a variety of compounds such as sugar,\(^10\)\(^-\)\(^19\) alcohol,\(^20\)\(^-\)\(^23\) formate (HCOO\(^-\)),\(^24\) and dihydrogen (H\(_2\))\(^25\)\(^-\)\(^37\) can be utilized as fuels for biofuel cells. In particular, H\(_2\)/dioxygen (O\(_2\)) and
HCOO⁻/O₂ biofuel cells can completely utilize the energy (in a two-electron oxidation of the fuels) with a high theoretical standard electromotive force (ca. 1.2 V). Some H₂/O₂ biofuel cells exhibit high power densities (8.4 mW cm⁻², 6.1 mW cm⁻²). On the other hand, formic acid is liquid at room temperature, is highly soluble in water, and can easily be handled, stored, and transported. By using formate dehydrogenases (FoDHs) and multicopper oxidases (MCOs) as catalysts for HCOO⁻ oxidation and O₂ reduction, respectively, a high-power and convenient biofuel cell can be constructed.

Formic acid is the first stable intermediate during the reduction of carbon dioxide (CO₂) that is known to be one of the major causes of the present global warming. It can be utilized not only as such an energy source for electric generation but as an energy and carbon source for microorganisms to produce high-molecular-weight hydrocarbons. In fact, a direct HCOO⁻ fuel cell has been demonstrated by employing a polymer anion exchange membrane and metal catalysts at the anode and cathode. In addition, an integrated electro-microbial conversion of HCOO⁻ from CO₂ to higher alcohols using Ralstonia eutropha H16 has been demonstrated. Therefore, we can propose a new energy strategy of C₁ chemistry utilizing the interconversion system between HCOO⁻ and CO₂ (Scheme 1). The general purpose of C₁ chemistry might be to convert molecules with one carbon atom such as carbon monoxide, methanol, formaldehyde, and methane into organic compounds with increased numbers of carbon atoms. However, our proposed C₁ energy strategy is based on the interconversion between CO₂ and HCOO⁻. An HCOO⁻/O₂ biofuel cell and a bioelectrocatalytic CO₂ reduction system with FoDH play central roles in the system. The new strategy utilizing enzymes as catalysts can generate energy and single-carbon feedstock under mild and safe conditions.

We have electrochemically studied tungsten (W)-containing formate dehydrogenase (FoDH1) from Methylobacterium extorquens AM1. FoDH1 was shown to produce MET-type bioelectrocatalytic currents for both HCOO⁻ oxidation and CO₂ reduction. In addition, FoDH1 exhibits low specificity to the second substrate, and the electron transfer rate constants between

Scheme 1.
A new energy strategy in C₁ chemistry
FoDH1 and mediators increase exponentially with an increase in the driving force complying with a linear free energy relationship. We have achieved a MET-type bioelectrocatalytic oxidation of HCOO$^-\$ at a high current density (145 mA cm$^{-2}$ at 60 °C) with low overpotentials using FoDH1 on a mesoporous carbon electrode. We have also constructed an efficient bioelectrocatalytic CO$_2$ reduction system with FoDH1 at a current density of about 17 mA cm$^{-2}$ under mild conditions (neutral pH, atmospheric pressure, 30 °C).

On the other hand, bilirubin oxidase (BOD) from Myrothecium verrucaria, which is one of the MCOs, is a promising enzyme for bioelectrocatalytic four-electron reduction of O$_2$ to water. BOD shows high bioelectrocatalytic activity even at neutral pH and a high formal potential close to that of the H$_2$O/O$_2$ redox couple. There have been many reports on the DET-type reaction at a variety of electrodes and some reports on the MET-type reaction, in which 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and some metal complexes have been utilized as mediators. When gaseous substrates are used (H$_2$, O$_2$, and CO$_2$), their low solubility often results in a diffusion-controlled reaction with low current densities. To solve this problem, a gas diffusion type electrode that can supply the substrate to an enzyme on the electrode from the gas phase has been proposed.

In this study, we attempted to construct a high-power HCOO$^-$/O$_2$ biofuel cell at neutral pH by combining an FoDH1-modified bioanode and a BOD-modified biocathode. To increase the power density, we have constructed efficient MET type reaction systems on mesoporous carbon electrodes.

2. EXPERIMENTAL SECTION

2.1. Chemicals.

Waterproof carbon cloth (WPCC, ECCC1-060T) was purchased from Toyo Corp. (Japan), and Ketjen Black (KB, EC300J) was kindly donated by Lion Corp. (Japan). Poly(1,1,2,2-tetrafluoroethylene) (PTFE, 6-J) fine powder was purchased from Du Pont-Mitsui Fluorochemicals Co., Ltd. (Japan). The PTFE membrane filter T050A025A (pore size 0.5 μm, thickness 75 μm) was obtained from Advantec (Japan). Poly(4-vinylpyridine) (PVP, average molecular weight 60000) and poly(ethylene glycol) diglycidyl ether (PEGDGE) were obtained from Sigma-Aldrich Co. LLC (USA). BOD (EC 1.3.3.5) from Myrothecium verrucaria was donated by Amano Enzyme Inc. (Japan) and used without further purification. FoDH1 was purified according to a literature procedure. Other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Japan), and all solutions were prepared with distilled water.
2.2. Electrode Preparation.

A gas diffusion type electrode was prepared as follows: KB powder (40 mg) was mixed with PTFE powder and homogenized in 3.5 mL of 2-propanol for 3 min at 0 °C to prepare a KB slurry (L = dm$^3$). Next, about 1.0 mL of the KB slurry was applied to one side of a 1.0 cm$^2$ piece of WPCC (as a platform of KB particles) and dried. The thickness of the KB layer was about 40 μm. This electrode is referred to as KB/WPCC. The PTFE membrane filter was attached to the opposite side (without KB) of the KB/WPCC electrode by pressure bonding to hold the electrolyte solution completely. For the bioanode, a gold film was prepared at a thickness of 80 nm by sputtering on the surface of the PTFE membrane filter (without KB/WPCC) on a desktop coater (SC-704, Sanyu Electron).

$N$-(4-Bromobutyl)-$N'$-methyl-4,4′-bipyridinium dibromide was synthesized according to a literature procedure. A viologen-functionalized polymer (VP) was prepared by dissolving $N$-(4-bromobutyl)-$N'$-methyl-4,4′-bipyridinium dibromide (1.1 g, 2.4 mmol) and PVP (0.5 g, 8.3 μmol) in 100 mL of $N,N'$-dimethylformamide, and the solution was stirred for 7 days at 45 °C. The product (VP) was precipitated in acetone and dried. A 2 μL aliquot of a 0.1 M phosphate buffer (pH 7.0) reaction solution containing 50 mg mL$^{-1}$ VP, 7 mg mL$^{-1}$ PEGDGE, and 7 mg mL$^{-1}$ FoDH1 was cast onto the surface of a glassy-carbon electrode (GCE, 3 mm in diameter, BAS) (M = mol dm$^{-3}$). The electrode was dried at 4 °C for 2 h. The electrode is referred to as FoDH1/VP/GCE. For the bioanode with a higher projected area, 60 μL of a 0.1 M phosphate buffer (pH 7.0) reaction solution containing 50 mg mL$^{-1}$ VP, 7 mg mL$^{-1}$ PEGDGE, and 7 mg mL$^{-1}$ FoDH1 was cast onto the surface of the KB/WPCC electrode. The electrode was dried at 4 °C for 2 h under reduced pressure. The electrode is referred to as FoDH1/VP/KB/WPCC.

A 0.3 mL aliquot of a BOD solution (20 mg mL$^{-1}$) dissolved in 10 mM phosphate buffer (pH 7.0) was applied to the surface of a KB/WPCC electrode. The electrode is referred to as BOD/KB/WPCC. ABTS modification of the KB/WPCC electrode was conducted as follows: a 160 μL aliquot of an ABTS solution (10 mM) was applied to a KB/WPCC electrode, which was previously dried at room temperature, and the surface was washed with 100 mM phosphate buffer (pH 7.0). The ABTS-modified electrode is referred to as ABTS/KB/WPCC. A 0.3 mL aliquot of a BOD solution (20 mg mL$^{-1}$) dissolved in a 10 mM phosphate buffer (pH 7.0) was applied to the surface of the ABTS/KB/WPCC electrode. The electrode was dried for 3 h under reduced pressure at room temperature. The electrode is referred to as BOD/ABTS/KB/WPCC.

2.3. Electrochemical Measurements.

All electrochemical measurements were performed using an ALS 704C electrochemical analyzer (BAS). A Pt mesh and a handmade Ag|AgCl|saturated KCl electrode were used as counter
and reference electrodes, respectively. All of the potentials are referenced to the reference electrode in this paper. A handmade gas diffusion type electrolytic cell identical with that reported in a previous paper was used for the measurements. The projected surface area of the working electrode was set to 0.27 cm². The measurements were performed under quiescent conditions in 0.1 and 1.0 M phosphate buffer (pH 7.0) at 40 °C under O₂- and Ar-saturated conditions.

2.4. MET-Type Biofuel Cell.

An MET type membraneless one-compartment biofuel cell was constructed by combining the gas diffusion biocathode (BOD/ABTS/KB WPCC) and the bioanode (FoDH1/VP/KB WPCC), as depicted in Figure 1. The projected surface areas of the biocathode and bioanode were adjusted to 0.27 cm² by covering the electrodes with a cell vessel, and the total volume of the electrolyte solution in the vessel was maintained at 1 mL. A Ti mesh was used as a current collector. The biocathode and bioanode of the cell were connected through a variable resistor (Type 2786 Decade Resistance Box, Yokogawa Electric Corp.). The cell voltage and potentials of the biocathode and the bioanode were measured with an electrometer (SC-7403, Iwatsu electric Co., Ltd.) at given values of the resistance in the range of 100 kΩ to 10 Ω. The current densities and power densities were calculated on the basis of the projected surface area of the electrodes. The measurements were performed in 1.0 M phosphate buffer (pH 7.0) at 40 °C under quiescent conditions.

Figure 1.
A schematic of the HCOO⁻/O₂ biofuel cell
3. RESULTS AND DISCUSSION

3.1. HCOO⁻ Oxidation at an FoDH1-Modified Electrode.

We synthesized a VP and immobilized it along with FoDH1 on a GCE. Figure 2A depicts cyclic voltammograms (CVs) for an FoDH1/VP/GCE in the presence and absence of HCOO⁻. The

![Figure 2A](image)

**Figure 2.**
(A) CVs for HCOO⁻ oxidation at an FoDH1/VP/GCE in 0.1 M phosphate buffer (pH 7.0) containing 100 mM sodium formate under Ar-saturated (black solid line) and atmospheric (grey solid line) conditions. The measurements were conducted at 40 °C under quiescent conditions. The scan rate (v) was 10 mV s⁻¹. The dotted line represents the CV in the absence of sodium formate. The inset shows the chronoamperogram. The electrode potential was −0.4 V. An Ar flow was stopped at a point indicated by the arrow (at 300 s).
(B) CVs for HCOO⁻ oxidation at an FoDH1/VP/KB/WPCC electrode in 1.0 M phosphate buffer (pH 7.0) containing 300 mM sodium formate at 40 °C under atmospheric and quiescent conditions. v = 10 mV s⁻¹. The dotted line represents the CV in the absence of sodium formate. The PTFE content was 20%.
midpoint potential of VP ($E_{m,VP}$) was −0.54 V, which was evaluated from the noncatalytic CV of VP (Figure 2A, dotted line). The sigmoidal curve represents the catalytic oxidation of HCOO−, in which FoDH1 and VP work as the catalyst and mediator, respectively (Figure 2A, black solid line). The current reached the limiting value at potentials higher than −0.5 V. The half-wave potential ($E_{1/2} = −0.54$ V) is in good agreement with the $E_{m,VP}$ value, which is only 0.1 V more than the formal potential of the HCOO−/CO2 couple ($E^\circ_{HCOO^-/CO_2,pH \gamma_0} = −0.64$ V). The catalytic current completely disappeared by KOH treatment of the bioanode, indicating that the bioelectrocatalytic HCOO-oxidation does not proceed without active FoDH1 (Figure 3). These results suggest that the VP-immobilized system can efficiently mediate the electron transfer from the immobilized FoDH1 to the electrode. In our previous works, we reported that such a polymer system seems to retain some extent of mobility, which is essential in MET-type bioelectrocatalysis.42

On the other hand, it may be expected that the reduced mediator generated in the FoDH1 reaction is reoxidized with dissolved O2, resulting in a decrease in the catalytic current. However, the presence of O2 has little influence on the CV of the catalytic oxidation of HCOO− (gray solid line and the inset in Figure 2A). This can be explained as follows: the thickness of the MET-type reaction layer is thinner than that of the enzyme mediator-immobilized layers, and the dissolved O2 diffusing to the outer surface of the immobilized layer is reduced by the reduced mediator generated in the FoDH1 reaction, in which HCOO− works as a sacrificial reagent. Since the concentration of HCOO− is sufficiently higher than that of dissolved O2, the catalytic current is hardly affected by the presence of

![Figure 3.](image)

Cyclic voltammograms (CVs) at a FoDH1/VP/GCE in 0.1 M phosphate buffer (pH 7.0) under Ar-saturated conditions (dotted line). The solid line is the CV on a KOH-treated FoDH1/VP/GCE containing 100 mM sodium formate under Ar-saturated conditions. Measurements were conducted at 40 °C under quiescent conditions. The scan rate (ν) was 10 mV s−1.
O₂. On the other hand, the presence of H₂O₂ also hardly affected the HCOO⁻ oxidation current (Figure 4). H₂O₂ generated in the auto-oxidation of the reduced mediator is further reduced to H₂O by the reduced mediator, and a similar protective effect was observed for H₂O₂ in the experiments. Such a protective effect of preventing an O₂-sensitive hydrogenase from an inactivation by O₂ was also reported for a redox hydrogel containing hydrogenase and viologen moieties. To increase the current density further, we employed the KB/WPCC electrode, because it has a higher surface area and a high porosity, allowing the high-speed transport of HCOO⁻ to the electrode. However, since the KB/WPCC electrode is a gas diffusion type electrode as used for high-power H₂/O₂ biofuel cells, gaseous O₂ in the atmosphere was reduced at the outside of the bioanode, resulting in a decrease in the HCOO⁻ oxidation current. Therefore, we utilized a bioanode with a thin Au layer on the PTFE membrane filter to prevent the O₂ penetration. The PTFE content (PTFE/PTFE + KB (wt %)) was 20%. The HCOO⁻ oxidation current density reached a value of 30 mA cm⁻² at −0.3 V (Figure 2B, solid line). Almost an identical value of the current density was obtained in chronoamperometric measurements during successive additions of formate (Figure 5). The E_{1/2} value (≈ −0.53 V) is practically in agreement with the E_{m,VP} value. The results indicate that the FoDH1/VP/KB/WPCC electrode is useful for the HCOO⁻ oxidation at high current density with low overpotentials under quiescent conditions.

Figure 4.

CVs for HCOO⁻ oxidation at an FoDH1/VP/GCE in 0.1 M phosphate buffer (pH 7.0) containing 100 mM sodium formate under Ar-saturated conditions in the absence (solid line) and presence (dotted line) of 0.87 mM H₂O₂. Measurements were conducted at 40 °C under quiescent conditions. v = 10 mV s⁻¹.
Figure 5.

(A) Time-course characteristics of the catalytic current density of HCOO\(^{-}\) oxidation on HCOO\(^{-}\) concentration \(c_{HCOO^{-}}\) at an FoDH1/VP/KB/WPCC electrode in 1.0 M phosphate buffer (pH 7.0) at 40 °C under atmospheric and quiescent conditions. The electrode potential was −0.2 V. At the points indicated by arrows, a sodium formate solution was successively added at final concentrations of 9.9, 48, 91, 130, 170, 230, 290, and 330 mM. The PTFE content was 20%.

(B) The dependence of the current density at −0.2 V on \(c_{HCOO^{-}}\).
3.2. O₂ reduction at a BOD-modified electrode at neutral pH.

BOD can work as a good DET-type bioelectrocatalyst for O₂ reduction at pH 5.0 using a variety of electrodes (such as mesoporous carbon electrodes). Figure 6A depicts CVs of BOD/KB/WPCC electrodes under O₂-saturated conditions. The PTFE content was 50%. The Faradaic waves are ascribed to the O₂ reduction because of the DET-type bioelectrocatalysis with BOD, as

(A) CVs at BOD/KB/WPCC electrodes in 1.0 M phosphate buffer (pH 7.0 (black solid line), and pH 5.0 (gray solid line)) under O₂-saturated and quiescent conditions. The dotted line represents the CV under Ar-saturated and quiescent conditions. The PTFE content was 50%.

(B) CVs at a BOD/ABTS/KB/WPCC electrode in 1.0 M phosphate buffer (pH 7.0) under O₂- (solid lines) and Ar- (dotted line) saturated conditions. The PTFE content was 20%.

Figure 6.
described in the literature. However, the BOD/KB/WPCC electrode at neutral pH exhibited a low catalytic current density, in comparison with that at pH 5.0. These results are coincident with the catalytic properties of BOD in solution. On the other hand, FoDH1 is not efficient at pH 5.0 because the optimum pH is in the basic range. Therefore, we have to construct a biocathode that works efficiently at neutral pH. To overcome this problem, we attempted to utilize ABTS as a mediator. In the MET-type reaction system, we can improve the catalytic current density easily to increase the concentration of the mediator. The BOD/ABTS/KB/WPCC electrode gave stable CVs during 100 cycle scans at a scan rate of 10 mV s⁻¹ under Ar-saturated conditions (Figure 7). The result indicates that ABTS was stably adsorbed on the KB without decomposition and desorption. Figure 6B depicts

![Figure 7](image1)

**Figure 7.**
Multi-scanned CVs at a BOD/ABTS/KB/WPCC electrode in 1.0 M phosphate buffer (pH 7.0) under Ar-saturated and quiescent conditions. 100 cycles were measured at 40 °C. \( v = 10 \text{ mV s}^{-1} \). The PTFE content was 20%.

![Figure 8](image2)

**Figure 8.**
CVs at a BOD/KB/WPCC electrode in 1.0 M phosphate buffer (pH 7.0) under O₂-saturated conditions in the presence (dotted line) and absence (solid line) of 10 mM ABTS. Measurements were conducted at 40 °C under quiescent conditions. \( v = 10 \text{ mV s}^{-1} \). The PTFE content was 20%.
CVs of the BOD/ABTS/KB/WPCC electrode under O$_2$-saturated conditions. The PTFE content was 20%. The appearance of a clear sigmoidal catalytic wave indicates that ABTS can function as a mediator in BOD-based MET-type bioelectrocatalysis for O$_2$ reduction (Figure 6B, solid line), as judged from the fact that the redox peak of surfaceconfined ABTS (Figure 6B, dotted line) changes to a steadystate sigmoidal wave on the catalytic CV (Figure 6B, solid line). The steady-state current density is in fact controlled by the gas permeability. The MET type catalytic current density was larger than that of DET type bioelectrocatalysis at neutral pH (Figure 6A, black solid line). The $E_{1/2}$ value was 0.48 V and is only 0.14 V more negative than the formal potential of H$_2$O/O$_2$ ($E^\circ_{H2O/O2,pH 7.0} = 0.62$ V$^{61}$). The results indicate that ABTS exhibits good characteristics as a BOD mediator in view of kinetics and thermodynamics even at neutral pH on a gas diffusion type electrode. On the other hand, a BOD/KB/WPCC electrode did not produce the MET type bioelectrocatalytic O$_2$ reduction current in the presence of ABTS dissolved in the electrolyte solution but only produced the DET-type bioelectrocatalytic O$_2$ reduction current (Figure 8). It is likely that the BOD-adsorbed layer prevents the approach of ABTS to the electrode surface. This is the reason for the adsorption of ABTS before BOD.

3.3. Construction of an HCOO$^-$/O$_2$ Biofuel Cell.

In general, DET-type biofuel cells do not need a separator because the enzyme absorbs on an electrode and there is no mediator in the system. On the other hand, MET-type biofuel cells often need a separator because of mediator leaking (or desorption) from the electrode, which causes serious crossover reactions: mediators desorbed from bioanodes will react at biocathodes or vice versa, leading to a decrease in the cell power by merely converting the redox reaction energy into heat. However, our HCOO$^-$/O$_2$ biofuel cell can work without separators because all enzymes and mediators are fixed stably on the electrodes. In addition, the presence of ABTS in solution has little effect on the catalytic current of the HCOO$^-$ oxidation (Figure 9). A similar protective effect has been shown in section 3.1. However, these protection reactions waste the fuel, HCOO$^-$, and we would utilize separators from the viewpoint of practical application. Considering the results described above, an HCOO$^-$/O$_2$ biofuel cell was constructed using the FoDH1/VP/KB/WPCC electrode as a bioanode and the BOD/ABTS/KB/WPCC electrode as a biocathode. The biofuel cell was operated in 1.0 M phosphate buffer (pH 7.0) containing 300 mM sodium formate under passive and quiescent conditions at 40 °C. O$_2$ gas was spontaneously supplied from the outside of the biocathode. Figure 10A shows the cell voltage (closed triangle), the biocathode potential (open circle), and the bioanode potential (closed circle) as functions of the current density, whereas Figure 10B shows the current density dependence of the cell power. The open-circuit voltage was 1.2 V. The value is close to the standard driving force of an ideal HCOO$^-$/O$_2$ cell (1.25 V). The current density increased with a decrease in the
voltage, and the maximum current density reached about 20 mA cm\(^{-2}\). The maximum power density reached 12 ± 1 mW cm\(^{-2}\) at a cell voltage of 0.78 V (note here that the errors in this study were evaluated using Student’s t distribution at a 90% confidence level). This value is much higher than those of other MET type biofuel cells in the literature. This result shows the great potential of the HCOO\(^-\)/O\(_2\) biofuel cell.

**Figure 9.**
CVs for HCOO\(^-\) oxidation at an FoDH1/VP/GCE in 0.1 M phosphate buffer (pH 7.0) containing 100 mM sodium formate under Ar-saturated conditions in the presence (dotted line) and absence (solid line) of 10 mM ABTS. Measurements were conducted at 40 °C under quiescent conditions. \(v = 10\) mV s\(^{-1}\).
Figure 10.

(A) Polarization curves of the biofuel cell at 40 °C. The cell voltage (closed triangle) and the potentials of the biocathode (open circle) and the bioanode (closed circle) are plotted as functions of the current density. The measurements were conducted in 1.0 M phosphate buffer (pH 7.0) containing 300 mM sodium formate under quiescent conditions. O₂ gas was supplied to the outside of the biocathode.

(B) The power density (P) is plotted as a function of the current density.
4. CONCLUSIONS

We have demonstrated a high-power HCOO\(^{-}\)/O\(_{2}\) biofuel cell. The cell utilizes a KB-modified waterproof carbon cloth as the electrode material. A gas diffusion type system was employed for the biocathode to realize high-speed substrate supply. For the bioanode, VP is a useful mediator for bioelectrocatalytic HCOO\(^{-}\) oxidation. For the biocathode, ABTS works as a good mediator at neutral pH on a gas diffusion type electrode. By combining the FoDH1/VP/KB/WPCC electrode and the BOD/ABTS/KB/WPCC electrode, we have succeeded in constructing a high-power HCOO\(^{-}\)/O\(_{2}\) biofuel cell based on MET type bioelectrocatalysis. The cell exhibited a power density of 12 mW cm\(^{-2}\) at a cell voltage of 0.78 V under quiescent conditions. To the best of our knowledge, the power density of our cell unit is the highest reported for the biofuel cells to date.

5. References


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2-3. Efficient bioelectrocatalytic CO\textsubscript{2} reduction on gas-diffusion-type biocathode with tungsten-containing formate dehydrogenase

Abstract

A new gas-diffusion-type biocathode was constructed for carbon dioxide (CO\textsubscript{2}) reduction. In this work, tungsten-containing formate dehydrogenase (FoDH1), which is a promising enzyme for interconversion of formate and CO\textsubscript{2}, was used as a catalyst and was absorbed on a Ketjen Black (KB)-modified electrode. We used 1,1’-trimethylene-2,2’-bipyridinium dibromide as a mediator, and the hydrophobicity of the FoDH1-absorbed electrode was optimized according to the weight ratio of the polytetrafluoroethylene binder to KB. We achieved cathodic current densities of about 20 mA cm\textsuperscript{-2} under mild and quiescent conditions (neutral pH, atmospheric pressure, and room temperature).

1. Introduction

The reduction of carbon dioxide (CO\textsubscript{2}) to generate reduced carbon compounds as fuels and chemical feedstocks is an essential requirement for a carbon-based sustainable energy economy [1–8]. An interconversion system of formate (HCOO\textsuperscript{-})/CO\textsubscript{2} is one of the strategies for this purpose. HCOO\textsuperscript{-} is the first stable intermediate during the reduction of CO\textsubscript{2} to methanol or methane and is increasingly recognized as a new energy source [9–12]. However, when CO\textsubscript{2} is reduced to HCOO\textsuperscript{-} directly on conventional electrodes, several byproducts are generated, and quite a high overpotential is required [8].

One of the most promising strategies for solving these issues is the utilization of enzymes as catalysts. Formate dehydrogenase (FoDH) generally catalyzes the oxidation of formate to CO\textsubscript{2}. On the other hand, certain FoDHs have been reported to act as CO\textsubscript{2} reductases, and there are several reports of bioelectrocatalytic CO\textsubscript{2} reduction with such FoDHs [13–20]. The enzymes allow the system to function for a specific biological reaction under mild and safe conditions, such as neutral pH, room temperature, and atmospheric pressure [13–20]. The electro-enzymatic devices can be used as energy conversion systems, such as efficient bioelectrochemical systems for CO\textsubscript{2} reduction. However, several problems still exist, such as low current densities and low stability. We have electrochemically studied tungsten (W)-containing formate dehydrogenase (FoDH1) from Methylobacterium extorquens AM1 [21]. It has been shown that FoDH1 produces mediated electron transfer (MET)-type bioelectrocatalytic currents for both of the HCOO\textsuperscript{-} oxidation and the CO\textsubscript{2} reduction. In the MET-type reaction system, artificial redox partners (mediators) enable enzymatic reactions to couple with electrode reactions by shuttling electrons between enzymes and electrodes. In addition, FoDH1 exhibits no specificity to the second substrate, and the electron transfer rate constants between the enzyme and mediators increases exponentially with an increase in the driving force. We have achieved...
a MET-type bioelectrochemical CO₂ reduction at high current densities and low overpotentials using FoDH1 [22].

A gas-diffusion-type electrode has been developed and employed for bioelectrocatalytic systems, such as efficient H₂ oxidation and O₂ reduction for biofuel cells [23–29]. On the other hand, electrochemical CO₂ reductions on various metals and metal compounds loaded in a gas-diffusion-type electrode were reported by several workers [30–34]. They achieved large current densities for CO₂ reduction, compared to electrolysis in aqueous electrolyte using metal electrodes.

In this study, we attempted to construct a new bioelectrocatalytic CO₂ reduction system under mild conditions. We focused on the introduction of a gas-diffusion-type electrode into an FoDH1-based bioelectrocatalytic system. From the viewpoints of the kinetics and the thermodynamics between FoDH1 and mediators [21], we used 1,1’-trimethylene-2,2’-bipyridinium dibromide (TQ; Fig. 1, inset) as a mediator. In addition, we focused on Ketjen Black (KB) as an electrode material to effectively immobilize FoDH1. This is the first report of a MET-type bioelectrocatalytic CO₂ reduction on gas-diffusion-type electrodes.

2. Experimental

2.1. Chemicals

Waterproof carbon cloth (WPCC, EC-CC1-060T) was purchased from Toyo Corp. (Japan), and KB (EC300J) was kindly donated by Lion Corp. (Japan). Poly(1,1,2,2-tetrafluoroethylene) (PTFE, 6-J) fine powder was purchased from DuPont-Mitsui Fluorochemicals Co. Ltd. (Japan). The PTFE membrane filter T050A025A (pore size: 0.5 μm, thickness: 75 μm) was obtained from Advantec (Japan). Other chemicals were obtained from Wako Pure Chemical (Japan) and all solutions were prepared with distilled water. FoDH1 was purified according to a literature procedure [21].

2.2. Electrode preparation

Gas-diffusion-type electrodes were prepared as follows: KB powder (40 mg) was mixed with PTFE and homogenized in 3.5 mL of 2-propanol for 3 min at 0 °C to prepare a KB slurry (L = dm³). Next, about 1.0 mL of the KB slurry was applied to one side of a 2.25 cm² piece of WPCC and dried. This electrode is called KB/PTFE/WPCC. The PTFE membrane filter was attached to the opposite side (without KB) of the KB/PTFE/WPCC electrode by pressure bonding to completely hold the electrolysis solutions. A 60 μL FoDH1 solution (20 μM of FoDH1 solution in 100 mM potassium phosphate buffer of pH 6.0) containing 0.5% (v/v) glutaraldehyde was applied to the KB/PTFE/WPCC electrode (M = mol dm⁻³). The electrode was dried for 4 h under reduced pressure at 4 °C.
2.3. Preparation of TQ

TQ was synthesized according to a literature procedure [18]. Briefly, 2,2’-bipyridine (10 mmol) was dissolved in 300 mL of acetonitrile and then 1,3-dibromopropene (100 mmol) was added to this solution and the reaction mixture was refluxed for 48 h. The yellow precipitate was collected by filtration and washed with acetonitrile. The desired product was dried under vacuum overnight.

2.4. Electrochemical measurements

Cyclic voltammetry and chronoamperometry were carried out on an ALS 611B electrochemical analyzer. A handmade gas-diffusion-type electrolysis cell identical to that reported in a previous paper [35] was used for measurements with some modification. The projected surface area of the working electrode for gas-diffusion-type electrolysis was set to 0.27 cm². A Ti mesh served as a current collector. A Pt mesh and a homemade Ag|AgCl|sat.KCl electrode were used as counter and reference electrodes, respectively. All potentials in this study are given with respect to the reference electrode. The measurements were performed in 0.1 M (pH 7.0) or 1.0 M (pH 6.5) potassium phosphate buffer at 30 ºC under quiescent conditions.

3. Results and discussion

3.1. MET-type Bioelectrocatalysis of CO₂ reduction with TQ in solution

TQ gave a pair of redox waves in cyclic voltammetry at a scan rate (v) of 20 mV s⁻¹ (Fig. 1). The formal potential of TQ was evaluated as $E^\circ_{\text{TQ}} = -0.74$ V (−0.54 V vs. SHE) as a mid-point potential in the cyclic voltammogram (CV) of TQ. In the presence of FoDH1, a large catalytic reduction wave with sigmoidal and steady-state characteristics was observed in 0.1 M potassium phosphate buffer (pH 7.0) containing 50 mM K₂CO₃ on a glassy carbon electrode (Fig. 1). The sigmoidal wave is due to typical mediated bioelectrocatalysis of the CO₂ reduction. The half-wave potential of the catalytic wave was close to $E^\circ_{\text{TQ}}$ and only 0.1 V more negative than the formal potential of the CO₂/HCOO⁻ couple ($E^\circ_{\text{CO₂/HCOO⁻}} = -0.64$ V, −0.44 V vs. SHE [8]). This means that the system achieves efficient CO₂ reduction with a very small overpotential.

In solution, the acid-base equilibrium and low diffusion coefficient of CO₂ become limiting factors when we aim to achieve a high current density with bioelectrocatalytic CO₂ reduction. A gas-diffusion-type electrode, which can supply a gaseous substrate from the gas phase, could be utilized to solve this problem. Given this hypothesis, we attempted to develop a new bioelectrocatalytic CO₂
reduction on a gas-diffusion-type electrode with FoDH1 and TQ.

![Graph](image)

**Figure 1.**
CVs on a bare glassy carbon electrode under Ar atmosphere in 0.1 M potassium phosphate buffer (pH 7.0) containing 3.2 mM TQ and 50 mM K₂CO₃ in the absence (solid gray line) and presence (solid black line) of 1.2 μM FoDH1. The dotted line is a background CV (before addition of TQ, K₂CO₃, and FoDH1). \( v = 20 \, \text{mV s}^{-1} \).

3.2. Bioelectrocatalytic CO₂ reduction on a gas-diffusion-type electrode

Fig. 2A shows CVs of the FoDH1-adsorbed KB/PTFE/WPCC electrode in 1.0 M phosphate buffer (pH 6.5) containing 10 mM TQ in a CO₂ or Ar atmosphere under quiescent conditions. The PTFE content was 20% (PTFE/PTFE + KB, w/w). The FoDH-adsorbed electrode did not give a significant cathodic wave in the absence of TQ under CO₂ atmosphere (inset of Fig. 2A). However, the faradic wave is ascribed to CO₂ reduction to HCOO⁻, due to MET-type bioelectrocatalysis with FoDH1 and TQ. A stable steady-state catalytic current density of \(-18 \, \text{mA cm}^{-2}\) was obtained after at least 60 s from chronoamperograms (CAs) at \(-0.8 \, \text{V}\) on the electrode. The current density corresponds to an HCOO⁻ production rate of 0.1 μmol cm⁻² s⁻¹. This is the first report of a gas-diffusion-type biocathode that is used for MET-type bioelectrocatalysis, and an enzyme-based bioelectrocatalytic CO₂ reduction system at such a high current density and low overpotential under quiescent and mild conditions. Unfortunately, the non-catalytic broadened wave of TQ (in the absence of FoDH1) indicates relatively poor interfacial electron transfer kinetics at the electrode.

The catalytic CV in Fig. 2A was recorded at the second scan. The steady-state anodic wave in a potential region more positive than \(-0.6 \, \text{V}\) is assigned to the bioelectrocatalytic oxidation of HCOO⁻ generated near the electrode surface during the cathodic reaction in the first and second scans. This indicates that FoDH1 can work as a MET-type bioelectrocatalyst for the interconversion of
HCOO$^-$ and CO$_2$. Since the oxidation is uphill due to a situation, in which $E^\circ_{\text{HCOO}} (-0.74 \text{ V}) < E^\circ_{\text{CO}_2, \text{pH}6.5} (-0.61 \text{ V})$, the reaction rate constant is small under the reaction conditions used, and a
steady-state current with relatively small current density was observed without concentration depletion of HCOO\(^{-}\) during the anodic scan.

In order to optimize the performance of the gas-diffusion electrodes, we need to consider several factors, such as the CO\(_2\) permeability and the hydrophilicity/hydrophobicity of the electrode. In this work, we optimized the PTFE content. The catalytic reduction current densities at the FoDH1-adsorbed KB/PTFE/WPCC electrodes with various contents of PTFE are summarized in Fig. 2B. The optimum PTFE content for the FoDH1-adsorbed KB/PTFE/WPCC was 20%, and the reduction current densities reached \(-17 \pm 1\) mA cm\(^{-2}\) (note here that the errors in this study were evaluated by the Student \(t\) distribution at a 90% confidence level). In addition, the value of the current density at \(-0.8\) V increased with the bulk concentration of TQ (\(c_{TQ}\)) in the manner of a Michaelis-Menten-type saturation curve (Fig. 2C). The apparent Michaelis constant against TQ was estimated to be \(2 \pm 1\) mM, the error being an asymptotic standard one in non-linear regression. We set \(c_{TQ}\) as 10 mM.

Fig. 2D shows the current response during continuous operation of the FoDH1-adsorbed KB/PTFE/WPCC. The current response decreased gradually with the time. The residual current response after 5 h continuous operation was 61%. The decrease is mainly due to a local pH change.
near the bio-three phase interface and the evaporation of the buffer solution by blowing CO₂

W-containing FoDH-catalyzed HCOO⁻ oxidation occurs at the W center of the enzymes [36]. This reaction does not involve oxygen atom transfer: the product of HCOO⁻ oxidation is CO₂ and not hydrogen carbonate, as was clearly demonstrated by the formation of ¹³C¹⁶O₂ gas during the oxidation of ¹³C-labeled formate in ¹⁸O-enriched water [37]. The FoDH1-catalyzed CO₂ reduction seems to follow the reverse reaction mechanism. Therefore, CO₂ binds to the reduced active site holding a protonated sulfo group, but not directly to the W atom. Given this hypothesis, having achieved such a powerful and efficient MET-type bioelectrocatalysis of CO₂ reduction with FoDH1, it would be effective to use CO₂ directly. However, the solubility of CO₂ in water is low (0.033 M at 25 °C under 1 atm [38]). In this study, we were able to overcome this issue by supplying gaseous CO₂ directly to the biocathode from the gas phase.

On the other hand, the optimum PTFE content was different from that determined in previous studies [27]. This is mainly due to the difference in the electron transfer-type of bioelectrocatalysis. In previous studies, a direct electron transfer (DET)-type reaction, in which enzymes can communicate directly with electrodes, was utilized. To achieve a large current density with MET-type bioelectrocatalysis, it is important to retain the mobility of enzymes and mediators to some extent [22]. We believe that the hydrophobicity of the electrode would affect the interaction between the adsorbed FoDH1 and soluble TQ. We need to consider the hydrophobicity of mediators and enzymes carefully when we optimize an electrode for MET-type bioelectrocatalysis on a gas-diffusion-type electrode.

4. Conclusion

We have constructed a new gas-diffusion system in the field of MET-type biocathodes for CO₂ reduction to HCOO⁻ using gaseous CO₂ directly at current densities of about 20 mA cm⁻² under mild conditions (neutral pH, atmospheric pressure, and room temperature). TQ was used as an efficient mediator for the CO₂ reduction. The control of the hydrophobicity of the FoDH1-absorbed electrode is an important factor in fabricating the gas-diffusion-type cathode system. The proposed bioelectrocatalytic system is very useful and effective for CO₂ reduction. Therefore, it certainly plays an important role in the field of green and sustainable chemistry.

5. References

Chapter 2, 2-3


Chapter 2, 2-3


Conclusions

In this work, the author succeeded in constructing a bioelectrocatalytic interconversion between CO$_2$ and formate with both DET-type and MET-type system. This system certainly play an important role in the field of green and sustainable chemistry.

In chapter 1, the author developed some methods to enhance DET-type bioelectrocatalysis of FoDH1. FoDH1 adsorbed on mesoporous electrodes realizes DET-type bioelectrocatalytic interconversion of the CO$_2$/HCOO$^-$ redox couple and of the NAD$^+$/NADH redox couple. This is the first report that an enzyme works as four-way bioelectrocatalysis. The occurrence of this bidirectional bioelectrocatalysis supports the idea that interfacial electron transfer proceeds with a relatively large rate constant, and that FoDH1 realizes fast uphill intramolecular electron transfer, most probably due to the large rate constants of the self-electron exchange reactions at the redox sites in FoDH1. To increase the DET-type activity of FoDH1, the author has developed a 4-mercaptopyridine-treated AuNP-embedded mesoporous carbon electrode as a new platform for DET-type bioelectrocatalysis of FoDH1. AuNPs 5 nm in diameter were embedded on KB/GCE. Microscopic measurements indicated that the AuNPs were uniformly dispersed on the KB surfaces without aggregation. We succeeded in enhancing the activity of DET-type bioelectrocatalysis of FoDH1 adsorbed on Py/AuNP/KB/GCE. Electrochemical measurements showed that the heterogeneous electron transfer between the FeSa site of FoDH1 and the surface of Py/AuNP/KB/GCE is electrochemically reversible. The electrochemically active site is reasonably assigned to an ion-sulfur cluster, which has a formal potential of $-0.591 \pm 0.005$ V that is independent of pH. The pyridine moiety on the mesoporous electrode plays important roles in enhancing the heterogeneous electron transfer and in increasing the probability of suitable enzyme orientation for DET reactions on the electrode. This work also suggests that a variety of redox enzymes can function as DET type bioelectrocatalysts on suitably tuned and tailored electrode surfaces. Because these DET-type bioelectrocatalytic signals reflect several physicochemical characteristics of the enzyme, these electrochemical methods will open a new avenue for the characterization of redox enzymes.

In addition, the author has demonstrated that FoDH1 can utilize several artificial electron acceptors for the HCOO$^-$ oxidation. Although the characteristics of the artificial electron acceptors are entirely different from that of the native electron acceptor, NAD$^+$, the reaction kinetics between FoDH1 and various electron acceptors including NAD$^+$ obey an LFER. This indicates that the interaction between FoDH1 and NAD$^+$ is not so specific. Therefore, the electron acceptors except NAD$^+$ can be utilized as mediators in bioelectrocatalysis of FoDH1. On the other hand, we have also reported that FoDH1 can catalyze the CO$_2$ reduction with MV and some other reduced dyes. When MV is utilized as a mediator, a two-way bioelectrocatalysis without overpotentials has been realized.
by controlling pH and the concentration of the substrates.

In chapter 2, we have constructed a high-power HCOO\(^{-}\)/O\(_2\) biofuel cell. The cell utilizes a KB-modified waterproof carbon cloth as the electrode material. A gas diffusion type system was employed for the biocathode to realize high-speed substrate supply. For the bioanode, VP is a useful mediator for bioelectrocatalytic HCOO\(^{-}\) oxidation. For the biocathode, ABTS works as a good mediator at neutral pH on a gas diffusion type electrode. By combining the FoDH1/VP/KB/WPCC electrode and the BOD/ABTS/KB/WPCC electrode, we have succeeded in constructing a high-power HCOO\(^{-}\)/O\(_2\) biofuel cell. The cell exhibited a power density of 12 mW cm\(^{-2}\) at a cell voltage of 0.78 V under quiescent conditions. To the best of our knowledge, the power density of our cell unit is the highest reported for the biofuel cells to date.

On the other hand, the author has constructed a new gas-diffusion system in the field of MET type biocathodes for CO\(_2\) reduction to HCOO\(^{-}\) using gaseous CO\(_2\) directly at current densities of about 17 mA cm\(^{-2}\) under mild conditions (neutral pH, atmospheric pressure, and room temperature). TQ was used as an efficient mediator for the CO\(_2\) reduction. The control of the hydrophobicity of the FoDH1-absorbed electrode is an important factor in fabricating the gas-diffusion-type cathode system. The proposed bioelectrocatalytic system is very useful and effective for CO\(_2\) reduction.
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List of Publications

Kento Sakai, Yuki Kitazumi, Osamu Shirai, Kazuyoshi Takagi, Kenji Kano
Direct electron transfer-type bioelectrocatalytic interconversion of carbon dioxide/formate and NAD+/NADH redox couples with tungsten-containing formate dehydrogenase

Kento Sakai, Yuki Kitazumi, Osamu Shirai, Kazuyoshi Takagi, Kenji Kano
Direct electron transfer-type four-way bioelectrocatalysis of CO$_2$/formate and NAD$^+$/NADH redox couples by tungsten-containing formate dehydrogenase adsorbed on gold nanoparticle-embedded mesoporous carbon electrode with 4-mercaptopyridine

Kento Sakai, Bo-Chuan Hsieh, Akihiro Maruyama, Yuki Kitazumi, Osamu Shirai, Kenji Kano
Interconversion between formate and hydrogen carbonate by tungsten-containing formate dehydrogenase-catalyzed mediated bioelectrocatalysis

Kento Sakai, Yuki Kitazumi, Osamu Shirai, Kenji Kano
Bioelectrocatalytic formate oxidation and carbon dioxide reduction at high current density and low overpotential with tungsten-containing formate dehydrogenase and mediators

Kento Sakai, Yuki Kitazumi, Osamu Shirai, Kazuyoshi Takagi, Kenji Kano
High-Power Formate/Dioxygen Biofuel Cell Based on Mediated Electron Transfer Type Bioelectrocatalysis

Kento Sakai, Yuki Kitazumi, Osamu Shirai, Kazuyoshi Takagi, Kenji Kano
Efficient bioelectrocatalytic CO$_2$ reduction on gas-diffusion-type biocathode with tungsten-containing formate dehydrogenase