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The Reversible Electrochemical Interconversion of Formate and CO₂ by Formate Dehydrogenase from *Cupriavidus necator*

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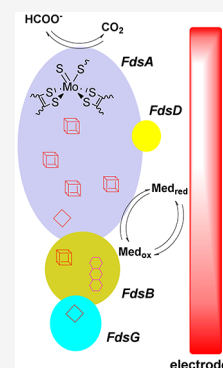


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ABSTRACT: The bacterial molybdenum (Mo)-containing formate dehydrogenase (FdsDABG) from *Cupriavidus necator* is a soluble NAD⁺-dependent enzyme belonging to the DMSO reductase family. The holoenzyme is complex and possesses nine redox-active cofactors including a bis(molybdopterin guanine dinucleotide) (bis-MGD) active site, seven iron–sulfur clusters, and 1 equiv of flavin mononucleotide (FMN). FdsDABG catalyzes the two-electron oxidation of HCOO[−] (formate) to CO₂ and reversibly reduces CO₂ to HCOO[−] under physiological conditions close to its thermodynamic redox potential. Here we develop an electrocatalytically active formate oxidation/CO₂ reduction system by immobilizing FdsDABG on a glassy carbon electrode in the presence of coadsorbents such as chitosan and glutaraldehyde. The reversible enzymatic interconversion between HCOO[−] and CO₂ by FdsDABG has been realized with cyclic voltammetry using a range of artificial electron transfer mediators, with methylene blue (MB) and phenazine methosulfate (PMS) being particularly effective as electron acceptors for FdsDABG in formate oxidation. Methyl viologen (MV) acts as both an electron acceptor (MV²⁺) in formate oxidation and an electron donor (MV^{•+}) for CO₂ reduction. The catalytic voltammetry was reproduced by electrochemical simulation across a range of sweep rates and concentrations of formate and mediators to provide new insights into the kinetics of the FdsDABG catalytic mechanism.



INTRODUCTION

Metal-dependent formate dehydrogenases are widely distributed in the microbial world and are found in bacteria and archaea.^{1,2} They bear either molybdenum (Mo) or tungsten (W) at their active site coordinated by the dithiolene side chains of two pyranopterin in their guanine dinucleotide form. The remainder of the metal coordination sphere consists of a protein-derived selenocysteine (Sec) or cysteine (Cys) plus a catalytically essential terminal sulfido ligand in a distorted trigonal prismatic geometry (Figure 1).³ All four combinations of metal (Mo or W) and protein ligand (Sec or Cys) have been observed in one or another formate dehydrogenase enzyme.^{1,4} A general observation is that the selenocysteine containing enzymes are more air-sensitive than their cysteine-containing analogues, but there are exceptions.⁵

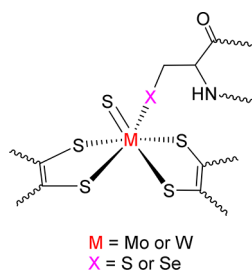


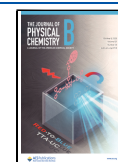
Figure 1. Modular active site of known metal-dependent formate dehydrogenase enzymes.

The physiological function of all formate dehydrogenases is the two-electron oxidation of formate (HCOO[−]) to carbon dioxide (CO₂) (eq 1) using various electron acceptors including NAD(P)⁺, cytochromes, quinones, and Fe–S proteins to mention but a few.^{3,4,6} It has emerged that most, if not all, formate dehydrogenases are in fact bidirectional and may catalyze the reverse reduction of CO₂ to HCOO[−].^{1,4} The preponderance of evidence is that the reaction occurs via hydride transfer from the C–H group of formate to form CO₂ directly,^{7,8} consistent with the earlier conclusion that CO₂ is the direct product of the reaction^{9–11} and that the reaction occurs without oxygen atom transfer. Still, mechanisms involving oxygen atom transfer have been proposed.^{12,13} At issue is whether CO₂ (eq 1) or HCO₃[−] (eq 3) is the immediate product of enzymatic formate oxidation.^{13,14} There are also structural studies that have revealed alternative active site structures to those shown in Figure 1 where dissociation of the protein ligand has occurred, at least under reducing conditions and dependent on the reductant.^{15,16} Regardless of the specific chemical mechanism, the reversible catalytic oxidation of formate/reduction of CO₂ continues to receive the attention of

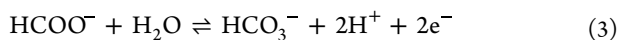
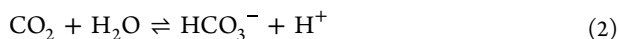
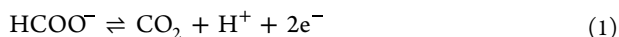
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researchers across diverse fields due to its relevance to global warming and also to future energy storage.¹⁷



The subject of the present study is FdsDABG formate dehydrogenase isolated from the facultative chemoautotrophic bacterium *Cupriavidus necator* (formerly known as *Ralstonia eutropha*). Like the closely related enzyme from *Rhodobacter capsulatus* whose structure has been determined by cryo-electron microscopy,¹⁸ FdsDABG is a dimer of heterotetramers and one of these is shown in cartoon form in Figure 2. The Mo

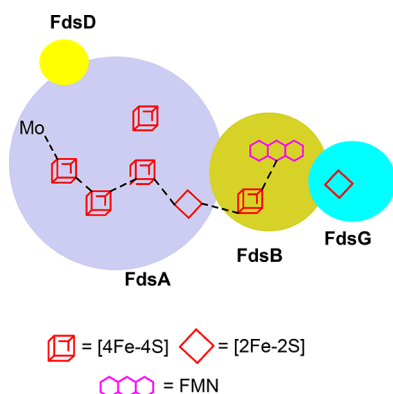


Figure 2. Cartoon representation of the *C. necator* FdsDABG heterotetramer, with its different subunits and cofactors indicated. The broken lines indicate the presumed electron transfer pathway from Mo to FMN following formate oxidation based on the structure of *R. capsulatus* FdsDABG.¹⁸

active site resides in the FdsA subunit along with four [4Fe–4S] clusters and one [2Fe–2S] cluster. The FdsB subunit contains a single [4Fe–4S] cluster and an FMN cofactor, while FdsG contains a [2Fe–2S] cluster. The smallest subunit FdsD bears no redox-active cofactors but is implicated in Mo cofactor insertion.¹⁹ The X-ray crystal structure of the FdsBG subcomplex from *C. necator* has previously been determined,²⁰ demonstrating that the FMN cofactor (of FdsB) is the site at which reduction of NAD^+ occurs, as is known to be the case in other members of the NADH dehydrogenase superfamily of enzymes, of which FdsDABG is a member.^{7,21–24} An unusual and convenient property of FdsDABG is its relatively high stability under aerobic conditions, although stabilizing inhibitors such as potassium nitrate or sodium azide are required for long-term retention of activity.²⁵

Electrochemical methods are well-suited to the study of oxidoreductase enzymes as the need for stoichiometric oxidants and reductants (e.g., NAD^+/NADH) is removed by the working electrode. Electrochemical communication with the enzyme may occur directly or indirectly via mediators which act as electron shuttles between the enzyme and the electrode surface. The first successful direct electrochemical investigation of a formate dehydrogenase was reported by Hirst and co-workers in 2008 for the complex W-dependent FDH1 from the sulfate-reducing bacterium *Syntrophobacter fumaroxidans*.²⁶ A key finding of this study was that, in the presence of both substrates, formate oxidation and CO_2

reduction were both observed as the potential was scanned in the positive or negative direction from the formal potential (-410 mV vs NHE at pH 7), respectively. This indicated that electrochemical communication with the W active site was very efficient and probably involved Fe–S clusters of the enzyme as relays. Subsequently, reports of electrochemical studies of other formate dehydrogenases including W-dependent FdhAB from *Desulfovibrio vulgaris* Hildenborough^{11,14,27,28} and *Methylobacterium extorquens* AM1^{29,30} have appeared. Similarly an electrochemical investigation of the Mo-dependent FDH-H (*Escherichia coli*)³¹ was reported.

This present study utilizes a selection of mediators of different redox potentials in partnership with *C. necator* FdsDABG which allows the driving force of both formate oxidation and CO_2 reduction to be controlled and thereby affect the overall reaction kinetics. The system has also been analyzed by simulation of the electrochemical data, shedding new insight on an earlier direct electrochemistry study³² of FdsDABG and its truncated constructs.

MATERIALS AND METHODS

Materials. Sodium formate, sodium bicarbonate (a surrogate for CO_2), chitosan, glutaraldehyde, phenazine methosulfate, methylene blue, and methyl viologen were purchased in their highest available purity from Sigma-Aldrich and used as received. All other reagents used were of analytical grade purity and were used as received. A chitosan solution (0.25% (w/v)) was prepared by dissolving chitosan flakes in 1% acetic acid solution under sonication for at least 2 h. Experiments at constant pH were carried out in phosphate buffered solution (100 mM, pH 7.0). All pH-dependent experiments were performed with mixed buffer systems comprising 20 mM of each citric acid, MES, Bis-Tris, Tris, and CHES, and the desired pH was obtained by the addition of dilute acetic acid or NaOH. All solutions were prepared with ultrapure water (resistivity $18.2 \text{ M}\Omega\cdot\text{cm}$).

Electrochemical Measurements and Electrode Cleaning. Cyclic voltammetry (CV) experiments were carried out with a BAS 100B/W or a BASi Epsilon Eclipse potentiostat. A three-electrode system was employed comprising a glassy carbon (GC) disk working electrode, a Pt wire counter, and a Ag/AgCl reference electrode calibrated with quinhydrone ($E' = +285$ mV vs the normal hydrogen electrode (NHE) at pH 7), and all applied potentials are cited versus NHE.

Experiments were carried out on solutions that had been purged with Ar gas for 30 min. The GC electrode was polished with 0.50 and 0.05 μm alumina slurry and then rinsed thoroughly with water. The electrode was sonicated in water for 10 min to remove adsorbed alumina particles and dried under a nitrogen atmosphere. The electroactive surface area of the GC electrode (A) was determined from cyclic voltammetry of 1 mM ferrocenemethanol in 0.1 M KCl solution at different sweep rates using the Randles–Sevcik equation (eq 4).³³

$$i_p = 2.69 \times 10^5 n^{3/2} A D_0^{1/2} C_0 v^{1/2} \quad (4)$$

The standard diffusion coefficient (D_0) of ferrocene methanol is $6.7 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$,³⁴ i_p is the current maximum, n is the number of electrons, C_0 is the concentration of analyte (mol cm^{-3}), and v is the sweep rate (V s^{-1}). The surface area was determined to be $A = 0.053 \text{ cm}^2$.

The variation of catalytic peak current at a constant potential (i_{lim}) as a function of substrate concentration was fit to

Michaelis–Menten kinetics (eq 5a)³⁵ yielding K_M^{app} (the apparent Michaelis constant) and i_{max} (the effective electrochemical turnover number defined in eq 5b where k_2 is the turnover number).

$$i_{\text{lim}} = \frac{i_{\text{max}}[\text{Substrate}]}{K_M^{\text{app}} + [\text{Substrate}]} \quad (5a)$$

$$i_{\text{max}} = 2FAk_2[\text{enzyme}] \quad (5b)$$

The pH dependence of the catalytic current was modeled by eq 6, which is applicable for an enzyme that is deactivated by either deprotonation of an acid at high pH (pK_{a1}) or protonation of a base at lower pH (pK_{a2}).

$$i_{\text{max}}(\text{pH}) = \frac{i_{\text{opt}}}{1 + 10^{(\text{pH}-pK_{a1})} + 10^{(pK_{a2}-\text{pH})}} \quad (6)$$

Enzyme and Electrode Preparation. *C. necator* FdsDABG was purified as described previously.²⁵ Immobilizing a small amount of active enzyme on the GC electrode surface as a thin layer can give rise to measurable voltammetry currents under turnover conditions when they are amplified by catalytic substrate oxidation/reduction processes. The integrity of the enzyme film is important for practical applications. Different immobilization strategies were pursued to obtain a stable FdsDABG enzyme film on the GC electrode using the cross-linker glutaraldehyde (GTA) and the biopolymer chitosan (poly-glucosamine). We obtained a very stable enzyme film when GTA alone was employed, but the catalytic currents were low compared to a GTA-free FdsDABG modified electrode. A similar observation was reported for other enzymes in the literature.^{36,37} However, the catalytic activity was notably improved by adding chitosan to the GTA-FdsDABG mixture, leading to a very stable and highly active film comprising a mixture of 2 μL of chitosan (0.25%), 2 μL of GTA (0.25%), and 3 μL of FdsDABG (86 μM). A similar strategy was used recently by our group to immobilize W-containing aldehyde oxidoreductase, and the resulting enzyme film exhibited excellent electrocatalytic activity along with stability.³⁸ The as-prepared electrode is abbreviated as GC/Chit+FdsDABG+GTA hereafter. The resulting modified electrode was stored at 4 $^{\circ}\text{C}$ in phosphate buffer solution (pH 7.2) when not in use. Unless otherwise stated, all of the experiments were performed under anaerobic conditions at 25 $^{\circ}\text{C}$.

Electrochemical Simulation. The DigiSim program (version 3.03b) was used to simulate the experimental cyclic voltammograms.^{39,40} The experimental parameters restrained in each case were the working electrode surface area (0.053 cm^2) and double-layer capacitance (6 μF). FdsDABG was effectively confined to the electrode surface within the Chit-GTA polymer network, whereas substrates and the mediators were under diffusion control. FdsDABG adsorbed on the GC electrode was modeled as a thin layer with blocked boundary conditions within DigiSim, and the thickness of the enzyme film was refined as 30 μm . The approximate theoretical concentration of FdsDABG within the film on the electrode surface is estimated to be 250 μM based on the number of moles of FdsDABG added to the film and the estimated thickness of the enzyme film. The redox potentials of methylene blue (MB) and phenazine methosulfate (PMS) were determined from control experiments in the absence of FdsDABG and formate. The diffusion coefficients of the mediators were also calculated by simulation of their cyclic

voltammetry currents at varying sweep rates in the absence of substrate and enzyme leading to values of 1.2×10^{-5} and $1.5 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ for MB and PMS, respectively. The diffusion coefficients for FdsDABG and formate were taken as 5×10^{-8} and $1 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, which are consistent with previous enzyme electrochemical investigations.^{41–43}

RESULTS AND DISCUSSION

Mediator Selection. In mediated enzyme electrochemistry, it is essential to match the redox potential of the mediator with the redox potentials of active redox cofactors present in the enzyme to obtain efficient intermolecular electron transfer. In the case of bidirectional enzymes such as FdsDABG, mediators also determine the potential bias toward either formate oxidation (high potential) or CO_2 reduction (low potential). The natural electron transfer partner for FdsDABG is NAD^+ , and its redox potential is -320 mV vs NHE at pH 7.²² As a substitute for NAD^+ , in the present study, we selected three synthetic redox mediators with different redox potentials, namely, methyl viologen (as its chloride salt, MV), methylene blue (as its chloride salt, MB), and phenazinium (as its methyl sulfate salt, PMS), to act as an artificial electron transfer partner for FdsDABG catalysis in place of NAD^+ (Figure 3). In

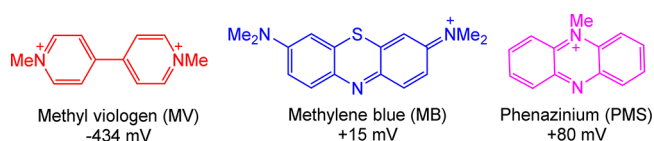


Figure 3. Organic electron transfer mediators and their redox potentials (pH 7, E' vs NHE) used in the present study.

other studies,^{44–46} MV has been used as a mediator for the CO_2 reduction reaction because of its pH-independent redox potential (-434 mV vs NHE) and its proximity to the $\text{CO}_2/\text{HCOO}^-$ redox potential (-410 mV vs NHE at pH 7).

Electrocatalytic Mechanism of FdsDABG. The mediated electrocatalytic mechanism for FdsDABG is shown in Scheme 1. It follows a two-substrate “ping-pong” mechanism comprising substrate binding (k_1/k_{-1}), turnover (k_2/k_{-2}), and reaction between the mediator and FdsDABG (k_3/k_{-3}). Formate oxidation or CO_2 reduction involves two electrons, and this affects the mechanism as one-electron transfer mediators must react twice (Scheme 1A) while two-electron acceptors react only once to restore the enzyme to its active form (Scheme 1B). The substrate and mediators are under diffusion control in solution, whereas FdsDABG is firmly entrapped in the Chit-GTA polymer network on the GC electrode surface.

Catalytic Formate Oxidation. No redox responses were observed upon immobilizing FdsDABG on the GC/Chit+GTA electrode in the absence of mediator. In the presence of 50 μM methyl viologen the enzyme electrode shows a well-defined quasi-reversible redox response at a midpoint potential of ca. -434 mV vs NHE (Figure 4A, red curve) which is expected behavior.⁴¹ The peak-to-peak separation (ΔE_p) is 110 mV, approximately twice that of a reversible single electron transient response indicating that heterogeneous electron transfer (k_0 , Scheme 1) is slowed by the Chit/GTA/FdsDABG film on the working electrode surface. Upon addition of 1.6 mM formate, the peak-shaped waveform of the $\text{MV}^{2+}/\bullet$ couple became asymmetric with a modest increase in the anodic current (Figure 4A, yellow curve). The enhancement of anodic

Scheme 1. Mediated Electrochemically Driven Catalysis of FdsDABG (Designated FDH Here) with (A) a Single-Electron Transfer Mediator and (B) a Two-Electron Transfer Mediator

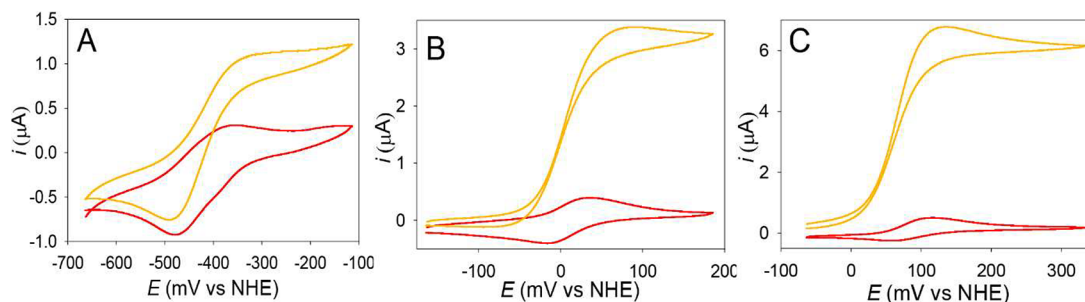
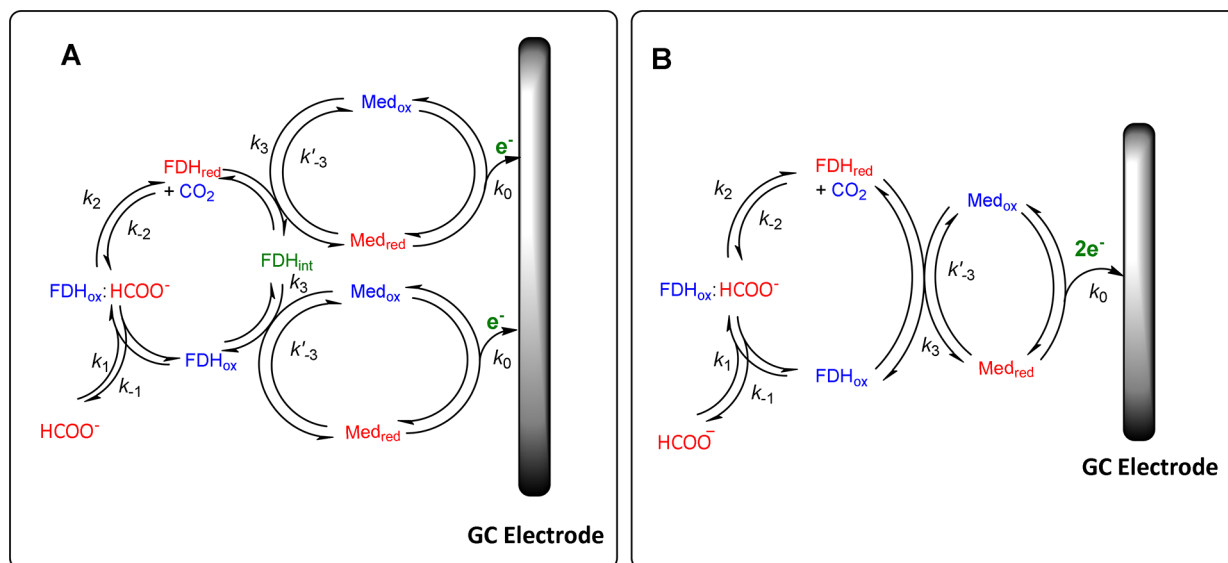


Figure 4. CVs obtained for (A) 20 μM MV, (B) 20 μM MB, and (C) 20 μM PMS at the GC/Chit+FdsDABG+GTA enzyme electrode in the absence (red curve) and in the presence (yellow curve) of 1.6 mM formate in 100 mM phosphate buffer solution (pH 7.0) at a sweep rate of 5 mV s^{-1} .

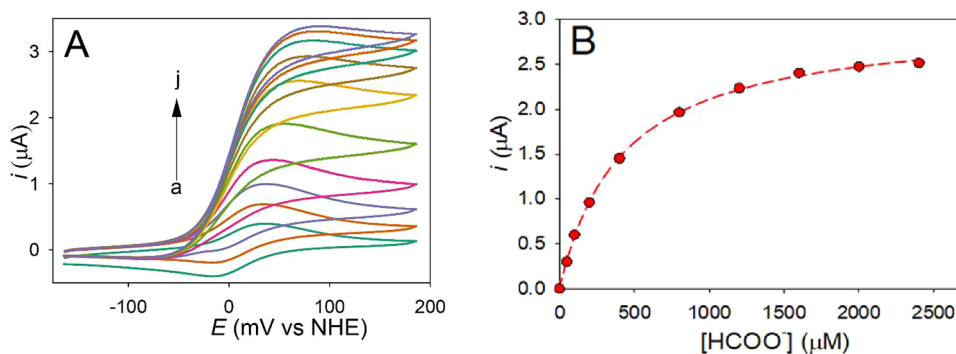


Figure 5. (A) CVs of 20 μM MB at the GC/Chit+FdsDABG+GTA electrode for varying sodium formate concentrations (a) 0, (b) 50, (c) 100, (d) 200, (e) 400, (f) 800, (g) 1200, (h) 1600, (i) 2000, and (j) 2400 μM at a scan rate of 5 mV s^{-1} in 0.1 M phosphate buffer solution (pH 7). (B) Plot of the baseline subtracted electrocatalytic anodic current at +50 mV as a function of the formate concentration.

current is indicative of a catalytic mechanism (EC_{cat}) where the one-electron reduced radical cation ($\text{MV}^{2+ \bullet}$) is regenerated by a homogeneous electron transfer reaction of MV^{2+} with partially reduced FdsDABG and electrochemical reoxidation of $\text{MV}^{2+ \bullet}$ is amplified.³³ In control experiments in the absence of FdsDABG, the redox response of $\text{MV}^{2+ \bullet}$ is unaffected by the addition of 1.6 mM formate (Figure S1), which confirmed that the magnified anodic (oxidative) current is linked to enzymatic formate oxidation by FdsDABG.

Similar CV experiments were conducted with the higher-potential mediators MB (Figure 4B) and PMS (Figure 4C). In the absence of formate (red curves), the peak-to-peak separations of the MB (ΔE_p 46 mV) and PMS (ΔE_p 45 mV) responses are both indicative of two-electron reactions, which is expected on the basis of their electrochemical properties. In the case of MB-mediated catalysis, after the addition of 1.6 mM formate to the electrochemical cell, a sigmoidal waveform emerges with a greatly enhanced anodic

current relative to the MV system. Moreover, no cathodic current is seen throughout the sweep (Figure 4B, yellow curve). The anodic and cathodic sweeps are almost superposed, which is consistent with an electrochemical steady state. For the PMS-mediated reaction (Figure 4C, yellow curve), the anodic current was twice that seen with MB. In addition, a peak was observed in the CV profile that is indicative of mass transport limitations, as will be discussed later. Clearly the electrocatalytic response of the enzyme electrode is very sensitive to the electrochemical driving force provided by the mediator.

Formate Concentration Dependence. An example is shown in Figure 5A for the substrate-concentration-dependent catalytic voltammetry of FdsDABG, where the formate concentration varies in the presence of a constant concentration of mediator (20 μ M MB). The quasi-reversible voltammogram of MB at the enzyme electrode (Figure 5A, green curve a) gradually transforms to first an asymmetric CV with enhanced anodic current upon addition of HCOO^- (Figure 5A, curves b–e) before becoming symmetrical and eventually independent of formate concentration (Figure 5A, curves i and j). Furthermore, the MB cathodic current diminished rapidly and was essentially zero beyond formate concentrations of 100 μ M indicating that the oxidized form of MB is consumed by (partially) reduced FdsDABG as rapidly as it is electrochemically regenerated at the electrode surface.

The same experiments were repeated with MV (Figure S2) and PMS (Figure S3). At low formate concentrations (<200 μ M) the transient waveform of MV persists and only transforms to an obviously catalytic and asymmetric waveform at formate concentrations greater than $\sim$ 800 μ M. In the case of PMS mediated catalytic voltammetry, complete transformation of the transient response of the mediator to a sigmoidal catalytic wave was more pronounced.

The resulting catalytic currents as a function of formate concentrations could be fit to eq 5a by Michaelis–Menten kinetics for MV (Figure S2B), MB (Figure 5B), and PMS (Figure S3B) mediated catalysis, yielding apparent Michaelis constants for formate oxidation (K_M^{app}) of 281, 416, and 898 μ M, respectively. Theoretically these values should be the same regardless of mediator, so these variations point to reactions other than substrate binding and turnover being significant. The published K_M (formate) is 310 μ M using NAD^+ as an electron acceptor.⁷

Mediator Concentration Dependence. The kinetics of the reaction between FdsDABG and its artificial electron acceptor was investigated by varying the mediator concentration. Figure 6 illustrates an example of the catalytic response of the enzyme electrode with varying mediator (PMS) concentrations in the presence of a saturating formate concentration. No catalytic current was seen in the absence of PMS in the potential window of -100 to $+400$ mV vs NHE (Figure 6, red curve a) as expected from a lack of non-turnover responses from any of the FdsDABG cofactors. However, upon introduction of 5 μ M PMS, a sigmoidal form of the catalytic wave emerged, which is indicative of an electrochemical steady state; the forward and backward sweeps are very similar. As the PMS concentration increases, the waveform becomes more peak-shaped, which is due to the mediator concentration being too high to sustain an electrochemical steady state. In other words, there is not enough reduced FdsDABG being formed (via formate oxidation) to sustain PMS in its fully reduced *leuco* form. A similar trend was observed for varying MB

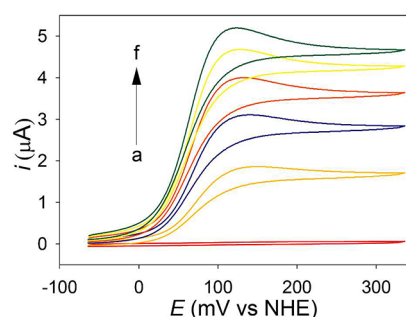


Figure 6. CVs obtained with 1.5 mM sodium formate at the GC/Chit +FdsDABG+GTA enzyme electrode for varying PMS concentrations (a) 0, (b) 5, (c) 10, (d) 15, (e) 20, and (f) 25 μ M at a scan rate of 5 mV s^{-1} in 0.1 M phosphate buffer solution (pH 7.0).

concentrations in the presence of a higher formate concentration, as shown in Figure S4.

pH Dependence of Formate Oxidation. The pH dependence of catalytic formate oxidation at the GC/FdsDABG electrode was explored in the range of $5.5 < \text{pH} < 10$. The prime intention of this experiment is to explore the pH-dependent activity of FdsDABG. Therefore, to avoid FdsDABG film stability issues on the GC electrode surface, the GC/FdsDABG electrode was securely covered with a dialysis membrane with a molecular weight cutoff of 3500 Da. Also, it is noted that the coadsorbents (chitosan and glutaraldehyde) were also omitted to avoid any unwanted pH-dependent interaction between these compounds and FdsDABG. Therefore, any variation in the catalytic current under these conditions is only attributed to the enzyme activation/deactivation process. Figure 7 depicts the plot obtained for

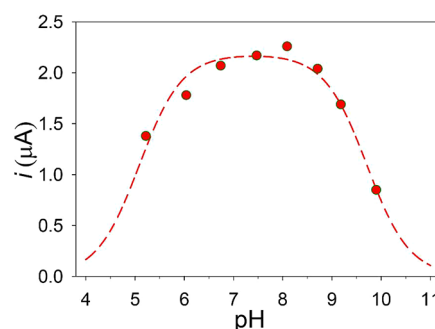


Figure 7. pH dependence of catalytic current for 20 μ M MB and 2.4 mM sodium formate at a GC/FdsDABG electrode (covered by a dialysis membrane). Scan rate of 10 mV s^{-1} in a 100 mM mixed buffer solution.

the baseline subtracted maximum catalytic current as a function of pH. The experimental CVs are listed in Figure S5. The catalytic current exhibits a pH optimum of 7.5, which is similar to that reported from kinetic assays performed for FdsDABG with the natural electron acceptor NAD^+ (7.5).²² A bell-shaped profile obtained by the application of eq 6 enabled the two pK_a values to be determined (5.1 and 9.7): the lower value defining the protonation constant of a base that switches off catalysis and the higher one associated with deprotonation of a catalytically essential acid. These pK_a values are also consistent with values obtained from solution assays ($\text{pK}_a = 5.6$ and 9.3).²² The pH profile was independent of the direction of titration, and the catalytic activity was essentially restored

when the solution pH was returned to its optimal value. It should be noted that the redox potential of the mediated catalytic voltammetry shifts to higher potential upon increasing the pH due to the pH dependence of the MB redox potential where proton transfer accompanies electron transfer (Figure S6). The electrochemical driving force and catalytic current may be somewhat affected by pH changes, regardless of acid–base reactions at the active site. However, it is also expected that the $\text{Mo}^{\text{VI/V}}$ and flavin (FMN) cofactor redox potentials, which are both coupled with proton transfer reactions, will also shift positively as the pH is lowered so the difference between the mediator and enzyme redox potentials most likely does not change with pH.

Catalytic CO_2 Reduction. It is challenging to reduce or activate CO_2 under ambient conditions due to its extreme thermodynamic and kinetic stability. Several metal-based (iron, ruthenium, manganese, silver, palladium, and copper) catalysts have been employed in the literature to reduce CO_2 using electrochemical methods.^{47,48} However, many of these systems are nonspecific (giving multiple products) and require high overpotentials, organic solvents, and high temperatures and pressures, which limits their practical applicability. Enzyme-based catalysis offers high specificity and function under ambient conditions and in neutral aqueous solution.^{49–51} So far, only two enzyme classes are known to be capable of reducing CO_2 , Ni-dependent CO dehydrogenase ($\text{CO}_2 \rightarrow \text{CO}$)⁵² and formate dehydrogenases isolated from different organisms ($\text{CO}_2 \rightarrow \text{HCOO}^-$).⁵¹

C. necator FdsDABG is in fact one such enzyme and is highly efficient in the catalytic reduction of CO_2 to HCOO^- ($k_{\text{cat}} = 11 \text{ s}^{-1}$) with NADH as the chemical reductant.²² NADH is a very expensive reductant, and an alternative dual enzyme (coupled) catalytic system has been devised to continuously regenerate NADH from NAD^+ while catalytically reducing CO_2 to formate. This involves glucose dehydrogenase which generates NADH as the product of glucose oxidation with FdsDABG utilizing equivalents of NADH for CO_2 reduction.²³

Figure 8 illustrates the electrocatalytic FdsDABG reduction of CO_2 with MV as the mediator in 0.1 M phosphate buffer solution (pH 7.0) at 25 °C. Here, the $\text{MV}^{+\bullet}$ radical serves as an electron donor for FdsDABG and NaHCO_3 is used as a surrogate for CO_2 . The concentration of CO_2 generated from NaHCO_3 varies depending on the experimental conditions,

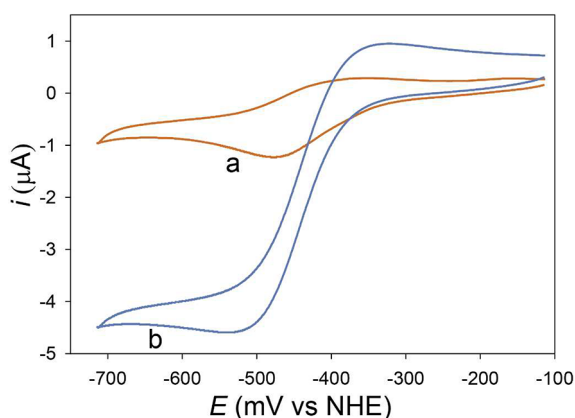


Figure 8. CVs of 50 μM MV in the (a) absence and (b) presence of 20 mM NaHCO_3 at the GC/Chit+FdsDABG+GTA electrode in 100 mM phosphate buffer solution (pH 7.0) at a scan rate of 5 mV s^{-1} .

such as the pH, temperature, and pressure. A 1 mM NaHCO_3 solution generates ca. 0.21 mM CO_2 under equivalent experimental conditions of (25 °C, 1 atm, and pH 7.0).⁵³ In the absence of NaHCO_3 , the FdsDABG modified electrode exhibited a quasi-reversible redox response at -434 mV with a peak-to-peak separation of 120 mV for 50 μM MV. Upon the addition of 25 mM NaHCO_3 ($\sim 5.27 \text{ mM}$ of CO_2), a sigmoidal cathodic reduction current emerged. In this case, electro-generated $\text{MV}^{+\bullet}$ acts as an efficient electron donor for FdsDABG to drive the catalytic CO_2 reduction.

Interestingly, an anodic formate oxidation current also emerged on the reverse sweep without formate being added to the solution. This is a consequence of electrochemically generated formate, from CO_2 reduction, accumulating at the electrode during the cathodic sweep which is then reoxidized on the anodic sweep by FdsDABG using MV^{2+} as the electron acceptor (as shown in Figure 4A). In this case, $\text{MV}^{2+/+\bullet}$ plays a dual role as an efficient electron acceptor or donor to reversibly interconvert formate and CO_2 . The CO_2 reduction reaction was also examined with benzyl viologen with a redox potential of -326 mV vs NHE that is ca. 100 mV more positive than the $\text{MV}^{2+/+\bullet}$ couple. FdsDABG produces a very weak catalytic response for CO_2 reduction in the presence of benzyl viologen (Figure S7). The low potential mediator MV is the only compound investigated here that is an efficient electron donor for FdsDABG-catalyzed CO_2 reduction.

The CO_2 concentration-dependent voltammetry of FdsDABG is shown in Figure S8. A pronounced cathodic current emerges with an increasing $\text{CO}_2/\text{HCO}_3^-$ concentration. The systematically increasing catalytic current followed Michaelis–Menten kinetics (eq 5a) and yielded an apparent Michaelis constant ($K_{\text{M}}^{\text{app}}$) of 860 μM , which is significantly lower than the value of $K_{\text{M}} = 2.7 \text{ mM}$ obtained from solution kinetic assays with NADH as the electron donor.²²

pH Dependence of CO_2 Reduction. The pH-dependent electrocatalytic current for CO_2 reduction at the GC/FdsDABG electrode is shown in Figure 9. The experimental

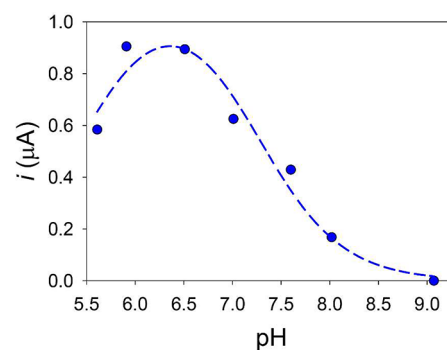


Figure 9. pH-dependent catalytic CO_2 reduction current magnitudes at the GC/FdsDABG electrode with 20 μM MV and 24 mM NaHCO_3 at a scan rate of 10 mV s^{-1} in a 100 mM mixed buffer solution.

data are given in Figure S9. An approximately bell-shaped profile is apparent, with a pH optimum of 6.5. Fitting the data to eq 6 gave pK_a values of 5.3 and 7.3 which are comparable with values reported by solution assays using NADH as an electron transfer partner (5.5 and 8.3).²² Inevitably the bicarbonate concentration will be sensitive to pH (eq 2) and should increase upon an increase in the pH of the solution. It was reported that the CO_2 catalytic efficiency of FdsDABG

decreased upon increasing the buffer ionic strength, which may also be reflected in these data.²²

Catalytic Interconversion of Formate and CO₂. Combining the above results from electrochemical formate oxidation and CO₂ reduction by FdsDABG, our aim was to observe the reversible electrocatalytic interconversion of formate and CO₂. Given that MB and PMS, with their high redox potentials, strongly bias the reaction in favor of formate oxidation, MV^{2+/+} was selected due to its redox potential being close to that of the HCOO[−]/CO₂ couple (−410 mV, pH 7). When both formate and bicarbonate are present in the bulk solution, catalytic anodic (formate oxidation) and cathodic (CO₂ reduction) currents are observed within the same cyclic voltammogram. Both reactions involve two electron transfer processes, so MV^{2+/+} must interact twice with FdsDABG in each sweep to accept/donate two electrons. As seen before, the *K_M* value for formate oxidation is an order of magnitude lower than that for CO₂ reduction so the formate and CO₂ concentrations were adjusted accordingly to compensate for the natural preference of FdsDABG toward formate oxidation. The midpoint redox potential of this catalytic wave is −420 mV, which is consistent with the thermodynamic potential of the HCOO[−]/CO₂ couple at pH 7. Other groups also used MV as an effective mediator for formate oxidation and CO₂ reduction with other enzyme systems. For instance, Kano and co-workers demonstrated the bifunctional activity of MV using W-dependent formate dehydrogenase (FoDH1) from *M. extorquens* AM1 with the enzyme in solution⁴⁶ and also immobilized on a Ketjen Black-modified electrode.³⁰

We observed different current saturation limits as a function of the HCO₃[−] concentration in the presence or absence of formate. As shown earlier, in the absence of formate, the catalytic cathodic current saturates at a HCO₃[−] concentration of ~24 mM (Figure S8). However, in the presence of added formate, the cathodic current saturates at a HCO₃[−] concentration of 8 mM (Figure 10). A recent study

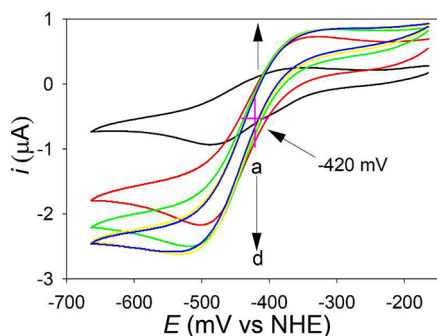


Figure 10. CVs for 50 μM MV²⁺ at the GC/Chit+FdsDABG+GTA electrode for respective formate and HCO₃[−] concentrations: (a) 0 and 0 (black), (b) 200 μM and 2 mM (red), (c) 400 μM and 4 mM (green), (d) 600 μM and 6 mM (blue), and (e) 800 μM and 8 mM (yellow) at a scan rate of 5 mV s^{-1} in 100 mM phosphate buffer solution (pH 7.0) and at 25 $^{\circ}\text{C}$.

demonstrated that *C. necator* FdsDABG-catalyzed CO₂ reduction was significantly inhibited by formate concentrations over 400 μM in the bulk solution.²³ Similar behavior would also explain the results shown in Figure 10 as a consequence of formate inhibition.

Stability of the FdsDABG Enzyme Electrode. The stability of the GC/Chit+FdsDABG+GTA enzyme electrode

was examined toward both HCOO[−] oxidation and CO₂ reduction, and results are given in Figure S10. Here, oxidized MB was used as the electron acceptor for HCOO[−] oxidation, and reduced MV was employed as the electron donor for the CO₂ reduction. FdsDABG was stable for HCOO[−] oxidation mediated by MB for 3 h under anaerobic conditions. Then, the electrode was removed from the electrochemical cell and stored in 100 mM phosphate buffer solution (pH 7.0) under aerobic conditions at 4 $^{\circ}\text{C}$ without any other additives. After a 24 h incubation, the GC/Chit+FdsDABG+GTA electrode retained only ~18% catalytic activity. Subsequently, the FdsDABG enzyme electrode lost all of its catalytic activity after 72 h (data not shown). These observations are consistent with a recent mechanistic study on air inactivation of *C. necator* FdsDABG.²⁵ It was shown that FdsDABG degraded under aerobic conditions due to attack by superoxide, resulting in desulfuration of the Mo=S active site and sulfite formation. However, FdsDABG may be protected from degradation by incubation with nitrate, which acts as an inhibitor. When the GC/Chit+FdsDABG+GTA enzyme electrode was stored in phosphate buffer containing 10 mM KNO₃ under aerobic conditions, there was virtually no loss of electroactivity over 24 h (Figure S10A).

In terms of CO₂ reduction, the electroactivity of the enzyme electrode showed a small but significant loss of the catalytic current for CO₂ reduction after standing for 15 min in a 10 mM NaHCO₃ solution (Figure S10B). The CO₂ concentration in the cell solution decreases irreversibly due to degassing. This could be reversed by a second addition of 10 mM NaHCO₃ that restored the original catalytic current.

Electrochemical Simulation. In recent years, we have employed simulation to better understand the mechanism of mediated enzyme electrochemical reactions.^{42,43,54,55} The objective of the simulation is to obtain rate constants defined in Scheme 1 that reproduce all voltammetric features over a range of sweep rates and substrate and mediator concentrations. The FdsDABG electrode exhibited a pronounced catalytic response for HCOO[−] oxidation with the two higher potential mediators, such as MB (+15 mV) and PMS (+80 mV), compared to the low potential mediator MV (−434 mV). Therefore, we focused on the mediators MB and PMS to simulate the catalytic formate oxidation electrochemistry. Also, both mediators are involved in two-electron transfer processes (Scheme 1B) which is convenient for comparing the catalytic efficiency of these mediators. The redox potential difference between these two mediators is around 65 mV so that they deliver significantly different driving forces for electrocatalysis. MB and PMS are quite similar, bearing the same charge and overall tricyclic aromatic structure.

Figure 11 illustrates experimental (solid line) and simulated (broken line) mediated catalytic voltammograms for 800 μM formate in the presence of 20 μM MB (Figure 11A) and PMS (Figure 11B). All other simulated CVs measured as a function of various mediators and substrate concentrations and scan rates are given in the Supporting Information (Figures S11–S33). A sigmoidal waveform for MB is seen at the lowest scan rate (5 mV s^{-1}) (Figure 11A). As the scan rate increases from 5 to 50 mV s^{-1} , the symmetric waveform transforms to an asymmetric peak-shaped curve. On the other hand, the higher potential mediator PMS maintains an asymmetric waveform from 5 to 50 mV s^{-1} , indicating a breakdown of the electrochemical steady state is caused by an excessive PMS concentration which exceeds the amount of electrons being

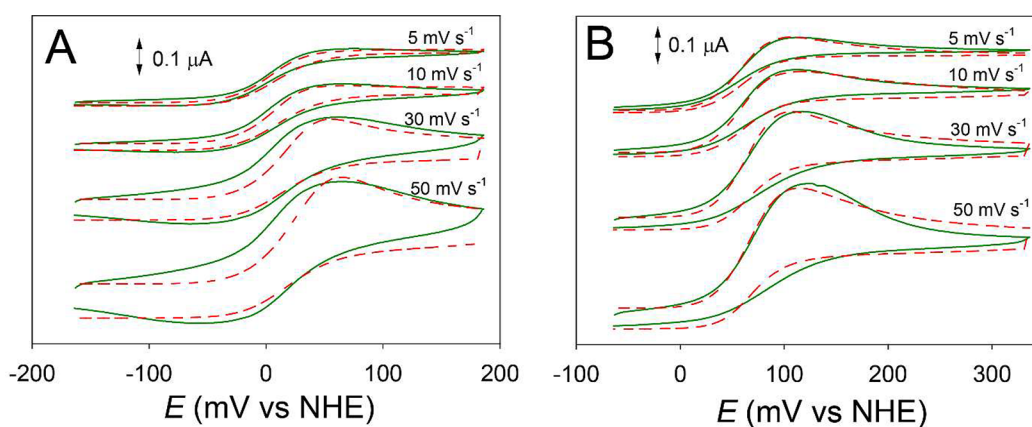


Figure 11. Experimental (solid lines) and simulated (broken lines) sweep-rate-dependent CVs obtained for (A) 20 μM MB and (B) 20 μM PMS in the presence of 800 μM sodium formate at the GC/CHIT-FdsDABG-GTA electrode in 100 mM phosphate buffer solution (pH 7) at different sweep rates.

provided by FdsDABG. All these catalytic wave transformation features were well reproduced in the simulation.

Analysis of Kinetic Parameters. The defined rate and equilibrium constants based on Scheme 1 are well-reproduced in all of the experimental voltammetry employed as a function of different scan rates, concentrations of formate, and two higher potential mediators such as MB and PMS. The same set of mediator-independent parameters (k_1 and k_2) was used for these two mediators. Different k_3/k_{-3} values (reaction between the mediator and FdsDABG) are expected and will be influenced by the redox potential and structure of the mediator which acts as an NAD^+ substitute in this system. Crystallography has shown that NADH/NAD^+ binds at the FMN site of the FdsB subunit,²⁰ and we presume that MB and PMS do likewise during catalysis. FdsDABG is capable of catalyzing the reverse reaction depending upon the redox potential of the mediator. Thus, the meaningful values of the reverse reaction rate constants (k_{-2} and k_{-3}) exist, but under these conditions the reaction is biased toward formate oxidation (k_2) and mediator reduction (k_3) so the k_{-2} and k_{-3} values in Table 1 are undefined but cannot be set to zero due to the requirement within Digisim that all chemical reactions are equilibria.

Table 1. Rate Constants Obtained for the FdsDABG Catalytic Reaction from Electrochemical Simulation^a

Parameters ^b	MB	PMS	
k_1 ($\text{M}^{-1} \text{s}^{-1}$)	1.7×10^3		Mediator-independent
k_{-1} (s^{-1})	0.68		
k_2 (s^{-1})	0.65		
k_{-2} (s^{-1})	4.0×10^{-4}		
K_M (μM)	782		
k_3 ($\text{M}^{-1} \text{s}^{-1}$)	5.4×10^4	2×10^5	Mediator-dependent
k_{-3} ($\text{M}^{-1} \text{s}^{-1}$)	0.02	0.05	

^aValues in italics are effectively zero under these conditions. ^b $K_M = (k_2 + k_{-1})/k_1$.

The precise estimation of multiple parameters is problematic due to the insensitivity of some parameters toward changing reactant concentrations and time scales (scan rates). The formate binding rate constant determined here ($k_1 = 1.7 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$) has not been reported for *C. necator* FdsDABG. However, the formate association rate constant value was reported for the formate dehydrogenase from *Pseudomonas sp.*

101 ($k_1 = 2.0 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$).⁵⁶ The somewhat lower k_1 value obtained here may be a consequence of access to the enzyme being slowed by the Chit-GTA polymer network. Nevertheless, the k_1 value is accurate under these conditions, and variations from its refined value had a significant effect on the quality of the fit especially at low formate concentration. The estimated turnover number ($k_2 = 0.65 \text{ s}^{-1}$) from the simulation is low compared to the value reported from the solution assay for *C. necator* FdsDABG with its natural electron acceptor NAD^+ (201 s^{-1}).⁷ However, as indicated above, this value should be viewed cautiously. As shown in eq 5b, the maximum catalytic current in each experiment at saturating substrate concentrations (i_{max}) is a product of two unknowns, the actual turnover number of FdsDABG within the Chit-GTA film (k_2) and the active enzyme concentration within the film. The active enzyme concentration within the film cannot be determined, but upper bounds can be set based on the amount of protein used and the estimated dimensions and volume of the enzyme film on the electrode. Various combinations of k_2 and $[\text{FdsDABG}]$ were trialed along with the depth of the enzyme film (another parameter within Digisim when using the “blocked boundary” model where the enzyme is confined to a thin layer before settling on a consensus value for each parameter). A lowered k_2 value indicates that the confined enzyme is less active than in solution, while the $[\text{FdsDABG}]$ value reports on the concentration of accessible enzyme within the film. It is expected that most of the FdsDABG protein is tightly entrapped by the chit-GTA polymer, and thus, the substrate can access only the FdsDABGs available on the top of the film facing the bulk solution.

The higher redox potential mediator PMS delivers a greater electrochemical driving force for electrocatalysis compared to MB. The outer sphere electron transfer rate constant (k_3) value was very sensitive toward various conditions such as lower and higher concentrations of HCOO^- and variations in mediator concentration. The calculated k_3 values in the present study are comparable to the reported value of $1.0 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ for the reaction between *C. necator* FdsBG complex and NADH .²⁰ Further, the estimated k_3 value for the PMS:FdsDABG reaction ($2 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$) is significantly greater than the value obtained for the MB:FdsDABG reaction ($5.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$) and it is consistent with predictions of Marcus theory ($\log k_3 \propto -(\Delta G^\circ)^2$).⁵⁷ The K_M value estimated from the

simulation rate constants [$K_M = (k_2 + k_{-1})/k_1$] for HCOO^- is somewhat higher (782 μM) than that reported from the solution assay (310 μM)⁷ with natural electron acceptor (NAD^+), but given the uncertainty surrounding k_2 mentioned above these differences are not substantial.

CONCLUSIONS

Mediated catalytic voltammetry of *C. necator* FdsDABG has been demonstrated in the presence of the artificial electron transfer mediators MV, MB, and PMS. The higher redox potential mediators MB and PMS act as efficient electron acceptors for HCOO^- oxidation. Interestingly, the low-potential redox dye MV acts as a bifunctional mediator that promotes both forward ($\text{HCOO}^- \rightarrow \text{CO}_2$) and reverse ($\text{CO}_2 \rightarrow \text{HCOO}^-$) reactions with a pronounced catalytic response. The pH-dependent catalytic activity of FdsDABG has been examined toward HCOO^- oxidation and CO_2 reduction, revealing bell-shaped profiles for both cases. Electrochemical simulation of the data has been performed to explore the kinetics of formate oxidation by FdsDABG mediated by MB and PMS, it being found that PMS was more reactive due to a higher driving force. The present results provide an avenue to construct an effective bioelectrochemical system for CO_2 reduction and formate oxidation.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jpcb.3c04652>.

Control experiments, substrate-concentration-dependent voltammetry not shown in the main text, and all comparative experimental and simulated electrochemical data at various scan rates and substrate and mediator concentrations (PDF)

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Notes

The authors declare no competing financial interest.

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