Disentangling the roles of free and cytochrome-bound flavins in extracellular electron transport from Shewanella oneidensis MR-1

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Abstract

In addition to its natural environmental significance, the process of extracellular electron transfer (EET) can be exploited at electrode interfaces to enable microbial electrochemical technologies for renewable energy recovery, biofuel production, bioremediation, and wastewater treatment. Success in these applications hinges on a better understanding of the electron transfer pathways that link electrochemically active microbes, such as *Shewanella oneidensis* MR-1, to electrodes. Here, we report electrochemical measurements that reveal both flavin-dependent and flavin-independent EET pathways from *S. oneidensis* MR-1. Significantly, differential pulse voltammetry captured the redox signatures of both free and cytochrome-bound flavins simultaneously in one experimental system for the first time, allowing us to compare their relative contributions while studying the impact of cell-removal, flavin addition, different culture conditions, and a mutant disrupted in flavin secretion. Our results indicate that endogenous flavins accelerate EET from *Shewanella* to high surface area carbon cloth electrodes primarily as cytochrome-bound cofactors, rather than free soluble shuttles, thereby supporting one of the two debated models of *Shewanella* EET. Our measurements also highlight the extent to which different electrode materials can impact detection of microbial redox signatures. This report motivates additional structural studies to study the binding location and precise interaction of flavins at cytochrome-electrode interfaces.

1. Introduction

Dissimilatory metal-reducing bacteria, including *Shewanella oneidensis* MR-1, gain energy by coupling the oxidation of organic fuels to the reduction of external electron acceptors ranging from natural minerals to electrodes [1, 2]. This respiratory strategy, known as extracellular

electron transfer (EET), is the focus of much fundamental and applied interest [3]. Microbes that perform EET are major players in global elemental cycles, with important consequences for climate change and bioremediation of toxic compounds and heavy metals [4]. And by interfacing their redox reactions to electrodes, such microbes can be used as biocatalysts in renewable energy technologies for converting the energy stored in diverse chemical fuels to electricity (microbial fuel cells), or vice versa (microbial electrosynthesis) [5, 6].

Shewanella oneidensis MR-1 is one of the most studied model organisms capable of EET. Since its discovery, extensive genetic, biochemical, structural, and electrochemical studies have resulted in multiple proposed mechanistic pathways that are typically categorized as direct or indirect, depending on whether cell-anode contact is required [7, 8]. For direct EET, MR-1 is proposed to route electrons from the inner membrane to extracellular acceptors through a network of periplasmic and outer membrane multiheme cytochromes that extend to the cellular surface [9-11] and along micrometer-long membrane extensions known as bacterial nanowires [12-14]. In this context, the MtrCAB porin-cytochrome complex is the primary route for electron delivery across the cell envelope, from the periplasmic decaheme cytochrome MtrA to the outer membrane decaheme cytochrome MtrC through the transmembrane porin MtrB [15]. For indirect EET, MR-1 is proposed to take advantage of naturally occurring or biogenic small molecules, such as secreted flavins (riboflavin and flavin mononucleotide) [16, 17], that diffusively shuttle electrons between cells and external solid electron acceptors. Despite the tendency to draw a rigid line between direct and indirect EET categories, the proposed molecular pathways (i.e. Mtr and flavins) are not mutually exclusive, may be operational at different conditions [18, 19], and can interact. For instance, while the MtrCAB pathway alone can mediate direct electron transfer from the interior of membrane vesicles to external minerals [11], structural studies have revealed

that the *Shewanella* outer membrane multiheme cytochromes contain flavin-binding sites [20, 21].

One hypothesis for defining the cytochrome-flavin interaction is that the Mtr pathway reduces secreted flavins that in turn function as indirect soluble mediators between MR-1 and external surfaces [22]. This view is consistent with the discovery of a bacterial flavin adenine dinucleotide exporter (bfe) necessary for high EET to glassy carbon [23], as well as measurements of high dissociation constants between cytochromes and flavins that suggest a fleeting interaction under oxidizing conditions [24]. A different hypothesis, supported by voltammetry studies in anaerobic conditions, invokes a more intimate interaction and the formation of a stable flavocytochrome where the flavins enhance EET as redox cofactors bound to the multiheme cytochromes MtrC and OmcA [25].

Edwards *et al.* recently reconciled these two seemingly disparate hypotheses by demonstrating that the *Shewanella* multiheme proteins can switch from cytochromes to flavocytochromes under anaerobic conditions, and that the switching mechanism is controlled by conserved redox-active disulfides that respond to oxygen presence [21]. The latter conclusions were based on genetic, spectroscopic, and structural measurements targeting the decaheme cytochromes MtrC, MtrF, OmcA, and UndA. In this study, we set out to electrochemically distinguish and quantify the contributions of diffusible flavins vs. cytochrome-bound flavin cofactors to the catalytic EET current (i.e. turnover conditions in the presence of an electron donor) between MR-1 cells and anodes, using voltammetric techniques. While the separate electrochemical signatures of free-form and bound flavins have been previously described for MR-1, a mechanistic understanding was hindered by the simple fact that previous experiments did not observe them simultaneously, making it difficult to compare their contributions to respiration. For example, while the high

sensitivity of differential pulse voltammetry (DPV) makes it an invaluable mechanistic tool, previous reports on MR-1 have either detected free-form flavins performing a two-electron redox reaction or cytochrome bound flavins facilitating a one-electron redox reaction, using glassy carbon or indium tin oxide (ITO) electrodes respectively [16, 25]. Our study disentangles multiple mechanisms by capturing the flavin-dependent (both soluble and cytochrome-bound) as well as flavin-independent (cytochrome hemes) routes of cell-to-anode EET in one experimental system, confirming that flavins enhance the catalytic current primarily as redox cofactors under our experimental conditions.

2. Materials and Methods

2.1 Cell Growth Conditions

Shewanella oneidensis MR-1 was grown aerobically in LB broth from a frozen (-80 °C) stock, at 30 °C, up to an optical density (OD₆₀₀ measured at a wavelength of 600 nm) of 2.4-2.6. From this preculture, 12 mL was pelleted by centrifugation at $4226 \times g$ for 5 min, washed three times, and resuspended in 600 mL of a defined medium containing 50 mM PIPES, 28 mM NH₄Cl, 1.3 mM KCl, 4.3 mM NaH₂PO₄·H₂O, 20 mM sodium DL-lactate as the electron donor, and 30 mM sodium fumarate as the electron acceptor (for anaerobic growth conditions). In addition, amino acid and trace mineral stock solutions were used to supplement the medium as described previously [26]. The pH of the medium was adjusted to 7.2 with NaOH. Cells were subsequently grown at 30 °C in the defined medium up to an OD₆₀₀ of 0.15 before being transferred to the bioelectrochemical reactor. The same growth procedure was used for the Δbfe strain lacking the bacterial flavin adenine dinucleotide exporter.

2.2 Bioelectrochemical Reactor

Four PW06 Carbon cloth (*Zoltek*, St. Louis, MO) working electrodes measuring 5 cm × 3 cm, a platinum wire counter electrode (*Sigma*, St. Louis, MO), and a Ag/AgCl reference electrode (1M KCl) (*CH Instruments*, Austin, TX) were fitted into a custom built bioreactor (600 mL volume). The working electrodes were sonicated in ethanol and distilled de-ionized (DDI) water before placement in the bioreactor, for cleaning and to enhance hydrophilicity. The working electrode potentials were maintained and monitored using a four-channel potentiostat (*eDAQ*, Colorado Springs, CO). Cultures were transferred to the reactor through a bench top pumping system (*Watson-Marlow*, Wilmington, MA), and anoxic conditions were maintained with constant purging of purified N₂ gas. After electrochemical cultivation and voltammetric measurements, the culture was removed from the reactor and the planktonic cells were separated from the medium by pelleting and repeated centrifugation of the supernatant (6085 × g for 15 min, repeated 15 times). The resulting cell-free spent medium (confirmed by plate counts) was transferred to a new bioreactor with the same size of pristine working electrodes for additional electrochemical measurements.

2.3 Electrochemical Measurements

The reactor's working electrodes, poised at 0.44 V vs. SHE using the four-channel potentiostat, were used as the sole electron acceptors (electrochemical cultivation) for 12 hours before voltammetric measurements. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed on the bioelectrodes with a Gamry 600 potentiostat (*Gamry*, Warminster, PA). CV was performed with a scan window of -0.36 to 0.54 V vs. SHE, using 1 mV/s scan rate. DPV was performed with the same scan window, 1 s period, 0.5 s pulse time, 50 mV pulse size and 1 mV step size. CV and DPV measurements were also performed on the spent medium

samples using the same parameters. In addition to the four technical replicates within each experiment, a minimum of three biological replicates was performed for each experimental condition reported here.

2.4 Microscopy

For fluorescence microscopy, carbon cloth fiber samples were first fixed in 200 μ L of fixation solution containing 2.5% glutaraldehyde, and then 1.25 μ g of FM 4-64FX (*Life Technologies*) membrane stain (dissolved in 10 μ L of deionized water) was added 20 minutes before microscopy. Imaging was done in the TRITC (*Nikon* filter set G-2E/C) excitation/emission channels with 500 ms exposure time. Electrodes were also examined using scanning electron microscopy (SEM) using a JEOL JSM 7001F field-emission microscope. SEM samples were subjected to a serial dehydration protocol using increasing concentrations of ethanol, and the dehydrated samples were then critical-point dried (*Tousimis Autosamdri 815, Tousimis Inc.*, Rockville, MD) ahead of SEM.

3. Results and Discussion

An anodic current was immediately observed following inoculation of MR-1 into the bioreactors containing working electrodes poised at 0.44 V vs. SHE (Fig. 1a). This current, which increased steadily to 2.5 ± 0.3 mA ($n=4$) after 12 hours, reflects the multistep oxidation of lactate and simultaneous EET to the electrode surface by cells, as previously observed [16, 27]. Following this period of electrochemical cultivation, the electrochemical interaction between MR-1 and the electrodes was further probed using cyclic voltammetry (CV) and differential pulse voltammetry. Within the -0.36 – 0.54 V (vs. SHE) CV potential scan range, two significant catalytic waves with onset potentials of -0.23 V and 0.20 V (vs. SHE) were observed under turnover conditions (Fig. 1b, black line). The observation of two redox pathways linking cells to the electrode is also

consistent with previous reports on MR-1 EET [19, 27-29]; the wave with an onset at -0.23 V reflects a flavin-dependent mechanism, while the higher-potential wave with an onset at 0.20 V is facilitated by direct EET through mutliheme cytochromes.

To test whether the flavin-dependent EET current stems from free secreted or cytochrome-bound flavins, the electrodes were subjected to DPV (Fig. 1b, red line). Compared to free-form soluble flavins, the redox reaction of cytochrome-bound flavins is expected to be shifted more than 100 mV in the positive direction [25]. Surprisingly, our DPV measurements revealed *both* these anodic peaks clearly with peak potentials (E_p) of -0.24 V and -0.11 V (vs. SHE), respectively, in addition to the expected peak assigned to outer membrane cytochromes at 0.21 V (vs. SHE). To further confirm the DPV peak assignments, reduced and degassed flavin mononucleotide (FMN) was added to the bioelectrochemical reactors to examine the impact on the three DPV peaks (Fig. 1c). The 0.21 V peak was unaffected by FMN addition, consistent with its interpretation as a cytochrome mechanism independent of flavins. As expected, the -0.24 V peak current increased linearly with FMN addition, consistent with its assignment to free-form molecular flavin. The -0.11 V peak current initially increased with addition of FMN, before saturation at 400 nM FMN, suggesting that the outer-membrane cytochrome binding sites become saturated at higher concentration. Importantly, the half-width potential of the -0.11 V peak is roughly twice that of the -0.24 V peak, in agreement with a previously suggested one-electron redox reaction *via* bound semiquinone, rather than the two-electron reaction for free FMN [25]. This DPV observation is also consistent with the derivative of the MR-1 cyclic voltammogram, where the cytochrome-bound flavin peak exhibited a 140 mV width (Fig. S1). Peak broadening beyond the theoretical Nernstian $90/n$ mV value of an ideal redox-active thin layer is expected for a heterogeneous biomolecular film containing both electrochemically inert components and

multiple redox species (in this case, additional redox molecules including different cytochromes and even multiple hemes within each cytochrome, all with a distribution of redox potentials and rate constants) and where background charging currents are present [30]. Both of these factors are at play in whole bacterial biofilms. Nonetheless, the broadening (rather than narrowing below 90 mV) further indicates a one-electron reaction for the bound flavins.

The observation of two clear flavin-dependent DPV peaks is significant, since previous DPV reports have featured either the free or cytochrome-bound flavin peak, depending on the electrode used, but not both. The free flavin DPV signature is typically detected using glassy carbon electrodes that have a high adsorption affinity for flavins, but generally lower surface area (i.e. compared to our carbon cloth) for cellular attachment, thereby favoring flavin-mediated EET [16, 31]. At the same time, direct EET through cytochromes and cytochrome-bound flavins has been detected using ITO electrodes, which have a low adsorption affinity for flavins [25]. Our use of carbon cloth electrodes, which offer both increased surface area for biomass attachment and high affinity for adsorbed flavins, is likely the reason for our observation of all previously proposed redox pathways in one experimental system. In this context, it is also important to note that, with this electrode material choice, CVs revealed equal contributions from the flavin-dependent and direct cytochrome-to-electrode pathways above ~ 0.3 V (Fig. 1b), in contrast to previous studies where the flavin-dependent mechanisms dominated. The ability to monitor all previously proposed redox pathways offered a valuable mechanistic tool, allowing us to compare the relative contributions of each pathway to turnover currents by studying spent media, different culture conditions, and a mutation affecting flavin secretion.

To distinguish between the biotic (cellular EET) and abiotic (e.g. soluble redox centers not necessarily linked to metabolism) contributions to the voltammetry currents observed, under the

same chemical environment, we now compare the CV and DPV profiles of MR-1 cultures to the spent media from the same cultures. Following CV/DPV measurements of MR-1, cells were removed by repeated centrifugation steps of the reactor contents, with the supernatant of each step collected for the next step (see section 2.2 for detailed conditions). The final spent media contained viable cell densities < 10 CFU/ml and were transferred to new bioelectrochemical reactors containing pristine working electrodes, identical to those used for live cell measurements. In contrast to the electrodes exposed to MR-1, where SEM and fluorescence microscopy (using the membrane stain FM 4-64FX) revealed uniform thin (submonolayer) biofilms, no evidence of cells were seen on electrodes exposed to the spent media, confirming that the latter were cell-free (Fig. 2).

Unlike the double-sigmoidal signatures obtained from turnover CVs of MR-1 coated electrodes, the spent-medium CVs showed only the classic peak-shaped signature, at -0.24 V, of the free flavin redox pair secreted into the medium by MR-1 (Fig. 3a, red line). Notably, the oxidation and reduction peaks are only separated by 8 mV, significantly smaller than the $59/n$ mV ($n=2$ for this two-electron reaction) predicted separation for a reversible diffusion-controlled process, indicating that the flavins are adsorbed to the electrode surfaces rather than diffusing from the bulk solution. Furthermore, the spent-medium DPV revealed only this free flavin peak at -0.24 V (Fig. 3b, red line). Significantly, the peak height of this abiotic measurement is identical to the measurement of the MR-1 coated electrode. Taken together, the absence of cytochrome and cytochrome-bound flavin DPV signatures in the spent medium (confirming that these redox centers are not secreted) and the unchanged free flavin redox peak height suggest that secreted flavins do not contribute to EET from MR-1 to electrodes. Rather, the flavin-dependent catalytic current observed with MR-1 (Fig. 1 and 3) is mediated by cytochrome-bound flavins [21, 25]

that function as redox cofactors. This conclusion is further reinforced by comparing the flavin-dependent CV catalytic wave to the position and magnitude of free and bound flavin peaks in DPV measurements and derivative CVs of MR-1. The midpoint potential (-115 mV) of the first CV catalytic wave coincides well with the DPV peak potential (-110 mV) of bound flavins [25, 32], rather than the more negative (-240 mV) free flavin peak (Fig. 1). The derivative CV (Fig. S1) revealed two major peaks corresponding to cytochromes (210 mV) and bound flavins (-130 mV), where the latter also coincided well with the catalytic wave's midpoint from CV. Free flavins were detected as a smaller peak in the derivative CV (at -210 mV, visible as a shoulder to the bound flavin signature), well negative of the catalytic wave's midpoint.

Varying the culture conditions of wild type MR-1 and analysis of the Δbfe mutant inhibited in flavin secretion [23] offered additional mechanistic insight. When MR-1 was pre-grown aerobically prior to inoculation into the reactors, the respiration current reached 400 μ A (only 16% of that of the anaerobically grown cells) and the DPV profiles showed a significant reduction in the cytochrome-bound flavin peak height, now only seen as a shoulder to the free flavin peak (Fig. 4a). This result is consistent with the conclusions of Edwards *et al.*, who noted that the *Shewanella* multiheme cytochromes can transition from less reactive cytochromes in aerobic cultures to flavocytochromes under anaerobic conditions [21]. When the electrochemical activity of Δbfe was tested in a manner identical to anaerobically grown MR-1 (Fig. 4b), its free flavin DPV signature was reduced relative to both MR-1 and its own cytochrome-bound peak, consistent with diminished (but not complete inhibition) of flavin secretion. Remarkably, however, the Δbfe cytochrome-bound DPV signature and overall catalytic currents observed by CV (Fig. 4b) are comparable to wild-type MR-1 (Fig. 1b). It is important to note that this outcome differs from previous observations of Δbfe respiration currents on glassy carbon

electrodes that are severely reduced when compared to MR-1 [23], again highlighting the effect of electrode materials on detection of microbial redox signatures, as discussed above. We further confirmed that observed differences stem from the electrode material choice by successfully replicating the reduced respiration currents of Δbfe using glassy carbon electrodes (data not shown). Taken collectively, the diminished free flavin profile of Δbfe , yet comparable catalytic activity and bound flavin signature relative to MR-1, support the conclusion that flavins accelerate EET as cytochrome-bound cofactors when interacting with higher surface area carbon cloth electrodes under our experimental conditions.

4. Conclusions

The described experimental system and electrochemical analyses simultaneously captured both the flavin-independent and flavin-dependent routes of EET between *S. oneidensis* MR-1 and electrodes. By studying the electrochemical signature of secreted redox-active molecules, different culture conditions, and a mutant disrupted in flavin secretion, our measurements indicate that flavins accelerate EET as cytochrome-bound cofactors, rather than free soluble molecular shuttles. Our studies also stress the impact of electrode materials and surface properties when defining specific microbial redox signatures. This work motivates further structural and biophysical studies to unravel the location and precise mechanistic pathway that allows cytochrome-bound flavins to mediate electron transfer at the interface of cellular proteins and electrodes.

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Figures

Fig. 1. Electrochemical analysis of *Shewanella oneidensis* MR-1 (a) Chronoamperometry of *S. oneidensis* MR-1 interacting with a carbon cloth working electrode poised at 0.44 V vs. SHE. (b) Turnover cyclic voltammetry (CV) reveals two catalytic waves corresponding to flavin-dependent and flavin-independent pathways (black line). Differential pulse voltammetry (DPV) adds additional detail, by simultaneously capturing two anodic flavin peaks (-0.24 V and -0.11 V, vs. SHE) corresponding to free and cytochrome-bound flavins respectively (red line). (c) Effect of adding flavin mononucleotide (FMN, final concentrations noted) on the DPV profiles. Schematics illustrate the assignment of DPV peaks to free flavins, cytochrome-bound flavins, and outer-membrane cytochromes.

Fig. 2. Microscopic study of electrodes with cell attachment and in spent medium (a) Scanning electron microscopy (SEM) of carbon cloth electrode fibers after electrochemical cultivation and CV/DPV measurements of *S. oneidensis* MR-1. Inset shows identical electrodes after electrochemical measurements on spent media, confirming no cell attachment. (b) Reflection mode optical microscopy (left) and corresponding fluorescence microscopy (right, using FM 4-64FX to stain cell membranes) of electrodes used in *S. oneidensis* MR-1 (top) and spent medium experiments (bottom).

Fig. 3. Voltammetric analysis. (a) CV comparison of *S. oneidensis* MR-1 cultures and spent media. (b) The corresponding DPV comparison of MR-1 cultures and spent media. MR-1 and spent media data are represented in black and red lines, respectively.

Fig. 4. Effect of culture conditions and disruption of flavin secretion on electrochemical activity
 (a) DPV of an aerobically pregrown MR-1 cells, showing a significant decrease in the cytochrome-bound flavin anodic peak (arrow). (b) CV (black) and DPV (red) of the Δbfe strain

lacking the bacterial flavin adenine dinucleotide exporter demonstrates a lower free flavin anodic DPV peak (-0.24 V) consistent with its decreased secretion into the medium, but comparable catalytic activity to MR-1 (shown in Fig. 1b).

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