

Flavin Redox Bifurcation

Flavin Redox Bifurcation as a Mechanism for Controlling the Direction of Electron Flow during Extracellular Electron Transfer**

Akihiro Okamoto,* Kazuhito Hashimoto, and Kenneth H. Nealson*

Abstract: The iron-reducing bacterium *Shewanella oneidensis* MR-1 has a dual directional electronic conduit involving 40 heme redox centers in flavin-binding outer-membrane *c*-type cytochromes (OM *c*-Cyts). While the mechanism for electron export from the OM *c*-Cyts to an anode is well understood, how the redox centers in OM *c*-Cyts take electrons from a cathode has not been elucidated at the molecular level. Electrochemical analysis of live cells during switching from anodic to cathodic conditions showed that altering the direction of electron flow does not require gene expression or protein synthesis, but simply redox potential shift about 300 mV for a flavin cofactor interacting with the OM *c*-Cyts. That is, the redox bifurcation of the riboflavin cofactor in OM *c*-Cyts switches the direction of electron conduction in the biological conduit at the cell–electrode interface to drive bacterial metabolism as either anode or cathode catalysts.

Iron-reducing bacteria, for example, *Shewanella oneidensis* MR-1, are capable of extracellular electron transport (EET), that is, the transport of electrons to extracellular insoluble electron acceptors such as metal oxides^[1] and anode electrodes,^[2] via several multi-heme proteins that function in unison as a biological “electron conduit” to transport electrons 20 nm or more across the outer membrane to the cell exterior.^[3] This biological conduit is also capable of transporting electrons into cells (that is, an extracellular cathode can serve as the electron donor to drive intracellular enzyme reactions^[4]). Thus, a single microbe can operate as either a reduction or oxidation electrode catalyst, with important implications for the impact of metal-reducing bacteria in geochemical mineral cycling^[5] as well as anaerobic iron corrosion,^[6] microbial fuel cells,^[7] and electrode biosynthesis.^[8] Therefore, determining the mechanism(s) that regulate the direction and extent of the electron flow constitutes a major challenge.

In *S. oneidensis* MR-1, the microbial EET process toward the anode utilizes the metal reducing (Mtr) pathway through which multi-heme *c*-type cytochromes (*c*-Cyts) transport electrons from the inner to the outer membrane *c*-Cyts (OM *c*-Cyts) MtrC and OmcA.^[3] MtrC and OmcA are approximately 80 and 90 kDa deca-heme *c*-Cyts, respectively, each with a single flavin-binding site in their extracellular space.^[9] Cell-secreted riboflavin (RF) and flavin mononucleotide (FMN) specifically associate with the OmcA and MtrC proteins, respectively, as redox cofactors to enhance the rate of the interfacial EET.^[10] While the electron transport mechanism from MR-1 to an anode is well understood,^[3b,10] the molecular-level details of the reverse reaction pathway remain unclear.^[11] We present whole-cell electrochemical studies of *S. oneidensis* MR-1 to compare the EETs that occur upon switching between the anodic and cathodic flows. Our data indicate that the bound RF cofactor in the OmcA protein extracts electrons from the cathode (Figure 1 a) and that the redox bifurcation of the bound RF dictates the direction of the electron flow at the cell–electrode interface (Figure 1 b).

It has been suggested that, as at the anode, the Mtr pathway and RF are also involved in the electron uptake of *S.*

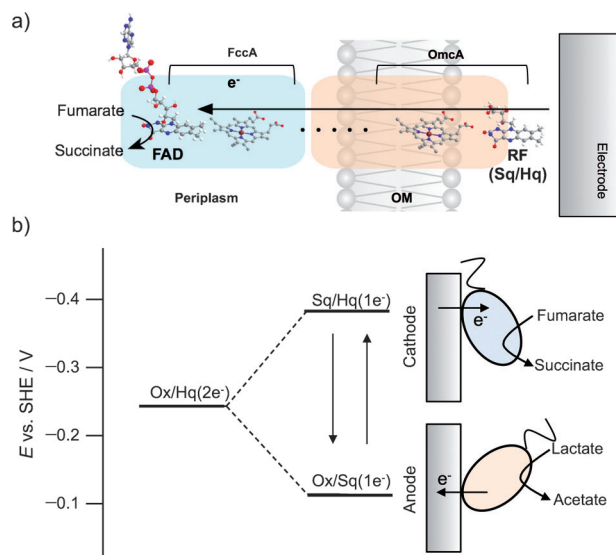


Figure 1. a) Illustration of a biological molecular conduit that transports electrons from an electrode to fumarate reductase (FccA) via an Mtr pathway initiated by the semiquinone/hydroquinone (Sq/Hq) redox reaction of RF at the electrode surface. b) Proposed model for switching the direction of the electron flow depending on the electrode potential owing to the flavin redox bifurcation of RF. The Sq/Hq extracts electrons from the cathode, and the oxidized/semiquinone (Ox/Sq) donates electrons to the anode in association with the metabolic reduction and oxidation reactions, respectively.

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oneidensis MR-1 from the cathode.^[11] However, the cathodic electron flow could affect the expression of the genes that encode the membrane proteins,^[12] and such proteins could be involved in the Mtr pathway, playing an important role in electron uptake and/or the manner in which RF is involved in EET. To examine whether cathodic EET requires additional proteins and how RF operates in EET under cathodic conditions, we first electrochemically examined the electron transfer process upon switching from anodic (electron donating) to cathodic electrode (electron uptake) conditions.

Shortly after the anodic cultivation of a monolayer MR-1 biofilm on the surface of an indium tin-doped oxide (ITO) electrode in a single-chamber three-electrode system as previously described (see the Supporting Information),^[10a,13] the MR-1 biofilm was subjected to an electrode potential of -0.45 V (vs. SHE) in anaerobic fresh media. Within 5 min, 50 mM fumarate was added as the sole electron acceptor, and cathode current production (j_c) was immediately observed (Supporting Information, Figure S1), indicating that no additional protein synthesis was required for electron uptake from the electrode.

In contrast, the redox properties of RF over the short duration of the cathodic experiment, as determined using linear sweep voltammetry (LSV) measurements, differed significantly from those under anodic conditions. As shown in the Supporting Information, Figure S2a, the onset potential (E_{os}) for the fumarate reduction current of MR-1 was -0.23 V. Upon the addition of $2 \mu\text{M}$ RF, the j_c curve with an E_{os} of -0.23 V immediately increased approximately 50-fold at -0.4 V (Supporting Information, Figure S2c). With sufficient fumarate, LSV monitors the potential dependency of the rate-determining EET step from the cathode to the cells.^[11] Thus, the observed j_c with an E_{os} of -0.23 V is attributable to the RF-mediated EET reaction. Because the E_{os} is located at -0.23 V even in the absence of exogenous RF, RF contributes significantly to the j_c , as in the EET process under anodic conditions.^[10] Remarkably, the redox potential (E_0) of RF at -0.4 V, approximated from the first-order derivative plot of the linear sweep (LS) voltammogram in the presence of fumarate, is distinct from the E_0 of RF at -0.1 V in the presence of lactate as the sole electron donor (Figure 2, inset). Furthermore, electrochemical studies have reported RF E_0 values of approximately -0.11 and -0.26 V for OmcA-bound RF under anodic conditions and a cell-free RF solution, respectively.^[10a,b] These significant differences in the E_0 values for RF indicate that cathodic conditions significantly alter the redox properties of RF. Considering that no additional protein expression in the Mtr pathway is required for cathodic EET upon switching from anodic conditions, these findings indicate that the mechanism underlying the switch in the direction of electron flow involves only the OM *c*-Cyts/electrode interface, most likely the redox properties of the flavin molecules.

It has been hypothesized that under cathodic conditions, the RF molecules shuttle electrons between OM *c*-Cyts and the electrode in a diffusing manner for microbial electron uptake via a two-electron redox reaction of soluble RF (RF (Ox) + $2e^- + 2H^+ \rightleftharpoons \text{RFH}_2$ (Hq)).^[11] However, the differences in the estimated E_0 values in the presence and absence of MR-

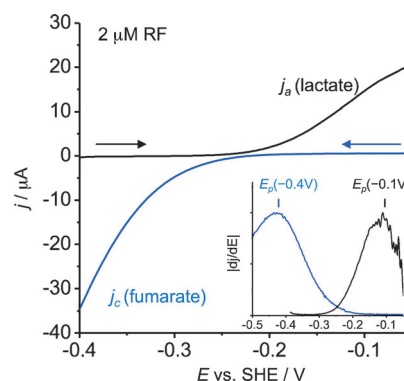
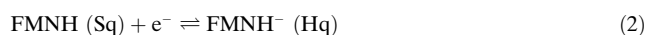
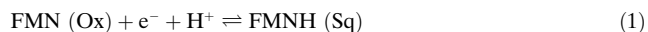


Figure 2. Linear sweep voltammograms of a monolayer biofilm of *S. oneidensis* MR-1 in the presence of $2 \mu\text{M}$ RF at a scan rate of 0.1 mVs^{-1} in the presence of 10 mM lactate (j_a) or 50 mM fumarate (j_c) as an electron donor or acceptor, respectively. Inset: the absolute value of the first derivative of j_a and j_c .

1 cells do not fit a shuttling model (Figure 2, inset). Instead, the negative E_0 for RF under cathodic conditions is consistent with our previous report that RF is stabilized as a singly reduced flavin semiquinone (Sq) upon binding with the OmcA protein scaffold as a cofactor to accelerate EET to the anode.^[10a,b] A similar mechanism occurs in another flavin-binding protein (flavodoxin),^[14] which can undergo two types of single-electron redox reactions to form the singly reduced Sq and doubly reduced hydroquinone (Hq) states of FMN, as described in Equations (1) and (2).^[14,15]



The redox potential of Sq/Hq [Eq. (2)] in the flavodoxins is approximately 300 mV more negative than that of Ox/Sq [Eq. (1)] at neutral pH.^[14] Because the E_0 of Ox/Sq is -0.11 V when RF is bound to the OmcA protein in MR-1,^[10b] the redox signal with an E_0 of -0.4 V in the inset of Figure 2 is assignable to the Sq/Hq redox cycling of the RF. This assignment explains why j_a and j_c exhibit little overlap of potential (Figure 2), because Sq should be reduced or oxidized in an equilibrium state at a given potential. Therefore, the Sq/Hq extracts the electron from the electrode to transport it to the periplasmic fumarate reductase (FccA) to drive fumarate reduction (Figure 1a). Furthermore, the switching between Ox/Sq and Sq/Hq is associated with the change in direction of the electron flow (Figure 1b). To confirm this hypothesis, we electrochemically monitored the redox cycling of RF in the presence of MR-1 cells in further detail under cathodic conditions and confirmed the interaction between RF and the OmcA protein using a mutant strain of OM *c*-Cyts.

Figure 3a depicts the differential pulse (DP) voltammogram of a monolayer biofilm in the presence of several RF concentrations with a redox signal at the peak potential (E_p) = -0.39 V. The peak current at -0.39 V ($j_{-0.39}$) was not observed in the absence of added RF, and it increased with the RF concentration (Figure 3a), indicating that the peak at -0.39 V is assignable to the redox reaction of RF. The half-

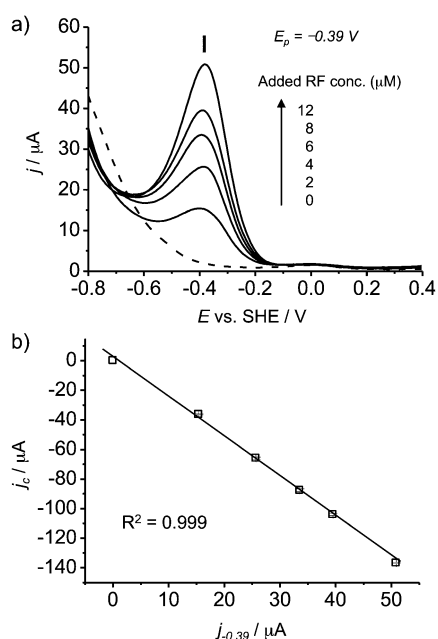


Figure 3. a) Differential pulse (DP) voltammograms for monolayer biofilms of MR-1 on an ITO electrode surface inoculated for 25 h at -0.45 V (vs. SHE) in the presence of 50 mM fumarate, in the absence of RF (.....), and in the presence of 2, 4, 6, 8, and 12 μ M added RF (—) under cathodic conditions. b) Plots of the bacterial current production (j_c) at an electrode potential of -0.45 V (vs. SHE) against the peak current ($j_{-0.39}$) of RF ($E_p = -0.39$ V vs. SHE). The squares of the correlation coefficients were estimated by adding the point of origin to the obtained data.

width potential ($\Delta E_{p/2}$) of the observed RF redox profile in the DP voltammogram indicates the number of electrons (n) involved in the redox reaction between RF and the electrodes.^[16] As indicated in Figure 3a, the $\Delta E_{p/2}$ value for RF at an E_p of -0.39 V was approximately 160 mV, which is nearly identical to that of the one-electron reaction (Ox/Sq) of OmcA-bound RF under anodic conditions.^[10b] While the two-electron redox reaction (Ox/Hq) of free RF provides a $\Delta E_{p/2}$ of approximately 60 mV (Supporting Information, Figure S3),^[10a,b] the observed $\Delta E_{p/2}$ value indicates that Sq was generated at the cell–electrode interface in the presence of the biofilms. To support the assignment of E_p at -0.39 V to the reduction of Sq to Hq, the effect of adding the free-radical scavenger tocopherol on the DPV signals was examined. Following the addition of 0.3 mM tocopherol to the system containing the monolayer biofilm, the peak E_p current at -0.39 V decreased by 30% (Supporting Information, Figure S4), indicating that the peak current was assignable to the reduction reaction of Sq to Hq.

Consistent with our assumption derived from the LSV data in Figures 2 and the Supporting Information, Figure S2, the contribution of the Sq/Hq redox cycling predominantly delivered electrons from the cathode to the cell. When we plotted j_c and $j_{-0.39}$ (Supporting Information, Figure S5 and Figure 3a, respectively), j_c exhibited a positive correlation with $j_{-0.39}$. A line fitted to these plots passed through the point of origin, yielding a square of the correlation coefficient of 0.999 (Figure 3b). Because the quantity of RF located at the cell–electrode interface correlated with the $j_{-0.39}$ in the DP

voltammograms, this result indicates that the observed fumarate reduction current is due to electron delivery to FccA via the redox cycling of RF at an E_p of -0.39 V.

Direct evidence for the interaction between OmcA and RF for a cathodic EET was obtained by measuring j_c and the DP voltammetry for mutant strains that lack the ability to produce OmcA ($\Delta omcA$) or MtrC ($\Delta mtrC$) in the presence of RF and fumarate. As indicated in Figure 4a, comparable current generation was observed for $\Delta omcA$ and $\Delta mtrC$ in the absence of added RF. However, upon the addition of RF to the reactor, a tenfold increase in current production was observed for the $\Delta mtrC$ strain, while far less enhancement was observed for $\Delta omcA$ (Figure 4a), indicating that OmcA proteins are necessary for RF to enhance the rate of cathodic EET from the electrode to the Mtr pathway. Furthermore, in the DP voltammogram for the $\Delta omcA$ monolayer biofilm (Figure 4b), a much smaller redox signal for RF was observed at an E_p of -230 mV, a value nearly identical with that of free RF. This significant effect of $omcA$ gene deletion on the E_p of RF suggests that the OmcA protein specifically interacts with RF at the cathode. Meanwhile, the redox signal of $\Delta mtrC$ was comparable to that of wild-type MR-1 (Figure 4b), further supporting our hypothesis that, in the $\Delta mtrC$ mutant, the OmcA protein associates with RF to deliver electrons from the cathode to the FccA protein via a heme conduit in the Mtr pathway (Figure 1a).^[11] Taken together, these data suggest that the RF bound in the OmcA protein scaffold mediates both anodic and cathodic EET, providing a mechanism that accounts for the switching of the electron flow from anodic to cathodic, that is, the redox bifurcation of the RF molecules from Ox/Sq to Sq/Hq (Figure 1b).

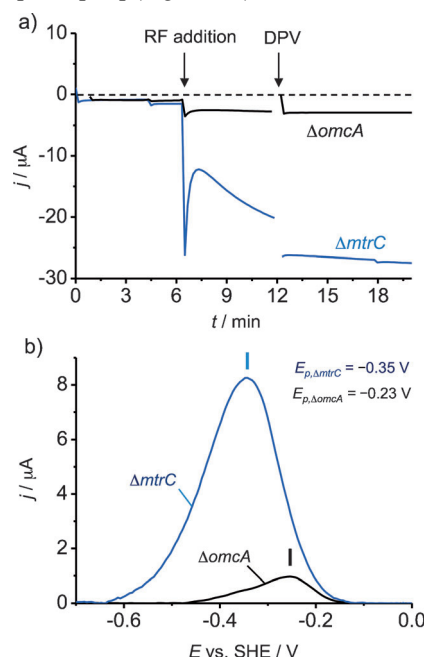


Figure 4. a) Cathodic current (j_c) vs. time (t) measurements for the $\Delta omcA$ and $\Delta mtrC$ strain of MR-1 cells in the presence of 50 mM fumarate on an ITO electrode surface at -0.45 V (vs. SHE). Arrows indicate the points at which 4.0 μ M RF was added to the reactor and DPV measurements were conducted. b) Baseline-subtracted DP voltammograms of RF in $\Delta omcA$ (black line) and $\Delta mtrC$ (blue line) in the presence of 4.0 μ M RF.

Reverse switching from cathodic to anodic conditions was associated with the generation of Ox/Sq redox cycling, as anticipated by our model (Figure 1b). After removing the fumarate-containing media, the addition of 10 mM lactate increased j_a (Supporting Information, Figure S6a). Furthermore, the addition of RF enhanced the j_a and Ox/Sq signals in the DP voltammogram (Supporting Information, Figure S6). These results further confirm the importance of RF redox bifurcation in the switching of electronic metabolism (Figure 1b). Furthermore, the other flavin species secreted by MR-1 cells, FMN, was unable to enhance the EET process upon switching from anodic to cathodic conditions. The addition of 4.0 μ M FMN provided a much smaller enhancement of the fumarate reduction current at -0.45 V compared with the addition of RF (Supporting Information, Figure S7a). Accordingly, the DP voltammogram in the presence of 4.0 μ M FMN exhibited no increase in a particular redox peak, in contrast to that observed with RF (Supporting Information, Figure S7b). By contrast, under anodic conditions, FMN produced a marked increase in current production via the redox cycling of the MtrC-bound FMN. Therefore, the lack of a redox signal is due not to a limited ability of FMN to diffuse in a monolayer biofilm but to the inability of the MtrC protein to associate with the FMN under cathodic conditions, which is presumably due to small structural perturbations induced by the negatively charged cathode surface.^[17] These results further support the requirement of a specific flavin, not non-specific free flavins, as a bound redox cofactor for activating the EET capability in the OM *c*-Cyt.

In conclusion, we demonstrated that the switching of microbial metabolism from lactate oxidation to fumarate reduction and vice versa is regulated by the chemically defined redox bifurcation of a RF molecule bound to the OM *c*-Cyt OmcA. The relatively negative potential of Sq/Hq is thermodynamically more favorable to prevent back electron donation from the heme center to the cathode (Figure 1b), which would facilitate electron uptake from various solids, even those with redox potentials more positive than -0.45 V. This chemistry-based mechanism allows the microbe to respond quickly to changes in the electrode potential or other charged solids, a reasonable strategy for microbes living on charged surfaces. Given this finding, it was not surprising to find the same ability in other metal-reducing microbes such as *Geobacter sulfurreducens*, a model microbe for microbial fuel cells and bioremediation.^[12,18] *G. sulfurreducens* is capable of reversing the direction of electron flow under cathodic conditions^[12] and utilizes flavins as redox cofactors in OM *c*-Cyts to mediate anodic EET processes.^[18] Thus, this mechanism may have major implications for both the biological chemistry of these iron-reducing bacteria and their application in microbial bioengineering and microbial electronics.

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