PNAS Classification**: (major)** Biological Science, **(minor)** Biophysics and computational biology

Electron transfer pathways

in a multi-heme cytochrome MtrF

*Hiroshi C. Watanabe [1,2], Yuki Yamashita [1], and Hiroshi Ishikita [1, 2]**

1) Department of Applied Chemistry, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-Ku, Tokyo 113-8654, Japan

2) Research Center of Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan

Running Title: Redox potential values in multiheme MtrF

CORRESPONDING AUTHOR: H. Ishikita, Research Center of Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan, Tel. +81-3-5452-5056, Fax. +81-3-5452-5083, E-mail: hiro@appchem.t.u-tokyo.ac.jp

ABSTRACT.

In MtrF, an outer-membrane multi-heme cytochrome, the 10 heme groups are arranged in heme-binding domains II and IV along the pseudo-C_2 axis, forming the electron transfer (ET) pathways. Previous reports based on molecular dynamics simulations showed that the redox potential (E_m) values for the heme pairs located in symmetrical positions in domains II and IV were similar, forming bi-directional ET pathways [Breuer et al. (2012) *J. Am. Chem. Soc.134*, 9868-9871]. Here we present the E_m values of the 10 hemes in MtrF, solving the linear Poisson-Boltzmann equation and considering the protonation states of all titratable residues and heme propionic groups. In contrast to previous studies, the E_m values indicated that the ET is more likely to be downhill from domain IV to domain II, due to localization of acidic residues in domain IV. Reduction of hemes in MtrF lowered the E_m values, resulting in switching to alternative downhill ET pathways that extended to the flavin binding sites. These findings present a novel explanation of how MtrF serves as an electron donor to extracellular substrates.

Significance

Cellular respiration process in dissimilatory metal-reducing bacteria is coupled to electron transfer. In *Shewanella* species, a decaheme cytochrome MtrF transfers electrons to extracellular insoluble substrates, such as Fe(III) and Mn(III/IV). Using the atomic coordinates of the MtrF crystal structure and analyzing interactions with the protein environments, we calculated the redox potential (E_m) values of the 10 hemes in MtrF. The E_m profiles show how the ET pathways proceed in MtrF. We demonstrated that when MtrF is reduced, the direction of the ET pathway switches, and bound flavin becomes the terminal electron acceptor.

\body

During cellular respiration in many gram-negative bacteria, ATP synthesis on the inner membrane is coupled to electron transfer (ET). In anaerobic conditions, dissimilatory metal-reducing bacteria can employ metal oxides, such as Fe(III) and Mn(III/IV), as the final electron acceptor (1). Because these metal oxides are membrane-impermeant, in *Shewanella* species multi-heme cytochromes transfer electrons to the metal oxides, e.g., a soluble decaheme cytochrome on the periplasmic side (MtrA), a hypothetical β-barrel porin in the trans-membrane region (MtrB), and a multi-heme cytochrome on the outer membrane (MtrC) (e.g., (2)). The 1.8-Å resolution crystal structure of MtrC shows that domains I and III are both β-barrel domains and are structurally similar (Figure 1) (3). These domains with the extended Greek key split-barrel structures are possible binding sites of flavin mononucleotide (FMN). The crystal structure of the extracellular decaheme cytochrome OmcA shows similar structural features (4).

MtrF is a homologue of MtrC, as confirmed by the crystal structure of MtrF at 3.2-Å resolution (5), and domains I and III flank the heme-binding domains (3, 5, 6). However, domain I contains only two β-sheets, whereas domain III is a β-barrel domain (Figure 1; see also figures in ref. (5)), a striking difference from the other decaheme cytochromes, MtrC and OmcA. In the crystal structure of MtrF, the 10 hemes are located in domains II and IV, forming ET pathways (Figure 1). Using the protein-protein interface server, Clarke et al. proposed that heme 10 is on the periplasmic side and heme 5 is solvent exposed (5). They also proposed that the two ET pathways that are terminated by heme 2 or heme 7 may function in reduction of FMN at the binding site (3, 7), whereas the ET pathway that is terminated by heme 5 may be used for direct reduction of extracellular insoluble substrates, e.g., Fe(III) (5). Insoluble substrates may also be reduced by FMN at the binding site (7, 8). In reduced MtrC, FMN showed pronounced binding affinity, compared to oxidized MtrC (3). The absence of the atomic coordinates of FMN in the MtrF crystal structure (5) implies that the crystal is in the oxidized state. In contrast, in the living system, hemes are likely to be in the reduced states due to continuously supplied electrons (9), which leads to pronounced FMN binding affinity and enhances the extracellular ET (10).

To understand the mechanism of ET in MtrF, the redox potential (E_m) values of the 10 heme groups must be determined. Although protein film voltammetry showed that the E_m values of the 10 hemes in MtrF range from −44 to −312 mV (5), specific values were not assigned to individual hemes. Breuer et al. calculated the E_m values using a thermodynamic integration (TI) approach based on molecular dynamics (MD) simulations (11). Breuer et al. uniformly added the constant C = −1567 mV (11) to reproduce the E_m range of −44 to −312 mV (5) reported for the hemes in MtrF by protein film voltammetry. Heme 1 (−41 mV) and heme 6 (−51 mV) in the middle of the ET chain had the highest E_m values, whereas heme 4 (−266 mV) and heme 9 (−279 mV) had the lowest E_m values, resulting in an ET chain energy profile that was essentially symmetrical (11). However, neither the amino acid sequences nor the locations of charged residues are highly conserved between domains II and IV (5) (Figure S1).

Here, we present the E_m values of the 10 hemes in MtrF, by solving the linear Poisson-Boltzmann equation and considering the protonation states of all titratable residues and heme propionic groups, in which the protonation states change in response to the heme redox states.

RESULTS

Structural disorder in domain I of the MtrF crystal structure.

In a split-barrel environment, the non-polar and polar residues are likely to alternate along the β-strands, with the non-polar residues oriented inward, forming the hydrophobic core, while the polar residues are exposed to the bulk solvent (e.g., Figure S2). Indeed, domain I of MtrF shows the alternating polar/non-polar pattern, as do other decaheme cytochromes. However, we found that in domain I of the MtrF crystal structure, the hydrophobic residues are oriented toward the bulk solvent (e.g., Leu50, Tyr66, Ile153, Tyr173, and Trp175), whereas the charged and polar residues are oriented toward the hydrophobic inner core (e.g., Asp65, Asn88, Arg150, Lys154, Asp174, and Gln176; Figures 3a and S1) (5). The MtrF crystal structure (5) shows few inter-strand H-bonds in the β-barrel domain, a striking difference from the crystal structures of other decaheme cytochromes (e.g., MtrC (3) and OmcA (4)). To evaluate the structural stability of the MtrF crystal structure, MD simulations were performed

before calculating the E_m for the hemes in MtrF. The MD simulations, performed using the original atomic coordinates of the MtrF crystal structure (5), suggested significant structural disorder specifically in domain I (Figure 2).

Next, we performed homology modeling as follows: (i) we constructed a sequential alignment with other decaheme cytochromes, to reproduce the proper orientations of the polar and non-polar residues and the location of the inter-strand H-bond in domain I of MtrF; (ii) we determined the atomic coordinates of domain I of MtrF, using domain I of the MtrC crystal structure at a resolution of 1.8 Å as a template (3) (Figure S1). Using the resulting homology model, we conducted MD simulations for structural refinement and verification. We found that the structural disorder of domain I, specifically that of the β-strands, was significantly decreased (Figure 2), and that the β-barrel structure of domain I was stable (Figures 3b and S3) during MD simulations. These results suggest that domain I of MtrF is highly likely to contain a β-barrel structure, as identified in the MtrC crystal structure (Figure 1b). Thus, we replaced domain I of the MtrF crystal structure with the one obtained by 1.0-μs MD simulation, and used the new structure for the following QM/MM calculations.

In the MtrF crystal structure, His ligands, e.g., heme 6 and heme10 (Figure S4) seemingly cause steric repulsion. The QM/MM calculations showed changes in the geometries of other heme groups in the MtrF. We replaced all 10 bis-histidine ligated *c*-type heme groups with the QM/MM-optimized geometry ("refined MtrF structure").

Effect of structural modifications on the E_m. The E_m values obtained by solving the linear Poisson-Boltzmann equation using the refined MtrF structure were almost the same (Table 1) as those for the MtrF crystal structure (Table S1) (5). Most modifications in the refined structure are in domain I, whereas domains II and IV remain unchanged except for the bis-histidine ligated *c*-type heme regions. These results suggest that domain I, which is not the heme-binding domain, did not significantly affect the calculated E_m values. Below, we refer to the E_m values calculated for the refined structure, unless otherwise specified (supporting data set 1 for atomic coordinates).

***E*m values.** Breuer et al. calculated the E_m for hemes in oxidized MtrF (11), where E_m for the focusing heme was obtained in the presence of the other 9 hemes being in the oxidized states (i.e., oxidized MtrF). In the present study, we calculated the E_m for both oxidized and reduced MtrF. The E_m values obtained solving the linear Poisson-Boltzmann equation were −47 to −336 mV for oxidized MtrF and −176 to −392 mV for reduced MtrF (Table 1). The calculated E_m shifts upon changes in the MtrF redox state are consistent with the E_m shifts observed in electrochemical analysis (9).These values are in the E_m range reported for MtrF based on protein film voltammetry, of −44 to −312 mV (5), or that reported for the MtrCAB complex, of 0 to −450 mV (13).

DISCUSSION

ET pathways. In contrast to the symmetric E_m profile reported by Breuer et al. (11), we obtained an E_m profile that indicated that the ET is more likely to be downhill from domain IV to domain II (Figure 4). In particular, among the heme pairs in domains II and IV, which are located at symmetrical positions with respect to the pseudo-C_2 axis, the (heme 4, heme 9) pair has the largest E_m difference (ΔE_m = 195 mV, Table 1) and the (heme 3, heme 8) pair has the second-largest E_m difference (ΔE_m = 167 mV). These E_m differences, i.e., low E_m values for heme 9 and heme 8 in domain IV with respect to heme 3 and heme 1 in domain II, are mainly caused by the acidic residues at Asp631, Asp518, Asp490 (in domain IV), and Asp377 (in domain III), specifically localized in domain IV (Tables 2 and 3); e.g., Asp631 decreases the E_m for heme 9 by −136 mV (Table S2). These acidic residues are not present in the corresponding regions of domain II. Although Breuer et al. also reported that Asp631 decreased the E_m for heme 9, the contribution was −1362 mV (11), which is unusually large (as discussed later).

The MtrF crystal structure shows that the Asp631 side chain is oriented toward heme 9 (~4 Å), which significantly decreases the E_m for heme 9 (Table 2). The E_m profile along the ET pathways remained downhill, even when titrated in the presence of protonated Asp631 (Figure S5). This suggests that the ET pathways could still be downhill, even if the orientation of Asp631 were disordered in the geometry

of the MtrF crystal structure, and protonated. The symmetric E_m profile along the ET pathways proposed by Breuer et al. (11) might be supported if the amino acid sequences of the heme-binding domains II and IV were similar. However, the amino acid sequence identity between domains II and IV of MtrF is low (23%, using ClustalW (14), Figure S1). Thus, each symmetrical pair of hemes is more likely to have different E_m values (Table 1), due to the contributions of different types of residues, as shown in Tables 2 and 3.

MtrF, MtrD, and MtrE are homologues of MtrC, MtrA, and MtrB, respectively. In the MtrCAB complex, MtrC has been reported to have higher E_m values than MtrA, based on electrochemical analysis using cyclic voltammetry (13), i.e., the ET pathway from MtrA to MtrC is downhill, while the ET pathway (Mtr pathway) can also mediate reversible ET (15). Considering the analogy between the MtrCAB and MtrFDE complexes (5), the corresponding ET pathway may proceed from MtrD to MtrF. It seems plausible that the ET pathway from domain IV to II is downhill, in terms of the location and function of MtrF, which is at the terminus of the intermolecular ET chain via the MtrFDE complex and directly reduces extracellular substrates via hemes 2, 5, or 7 (5, 6). It should also be noted that ET occurs also in the uphill ET pathway (e.g., the cytochrome *c* subunit of photosynthetic reaction centers from *Blastochloris viridis* (16)); this also suggests that a completely symmetric E_m profile (e.g., (11)) is not necessarily required to facilitate the reversible ET (15) in the Mtr conduit.

Switching the ET pathway in response to the MtrF redox state. In oxidized MtrF, the E_m values for hemes increase along the chain of hemes 9, 8, 6 (domain IV), 1, and 3 (domain II), resulting in a downhill ET pathway, [hemes 9 → 8 → 6 → 1 → 3] (Figure 4). In reduced MtrF, the E_m values were significantly lower, switching the ET pathway to [hemes 9 → 8 → 6 → 1 → 2] or [hemes 9 → 8 → 6 → 7] (Figure 4).

Intriguingly, (i) in reduced MtrC, FMN showed pronounced binding affinity, compared to oxidized MtrC (3). (ii) Hemes 2 and 7 have been proposed to be located near the FMN binding site (5, 6). (iii) The E_m of bounded FMN is not known for MtrF, but for MtrC it is reported to be ca. –150 mV using

differential pulse voltammetry (8, 17). If this holds true for MtrF, bound FMN can serve as an electron acceptor for both hemes 2 and 7 at the terminus of the entire ET pathway (–176 and –185 mV, respectively; Table 1) when MtrF is reduced (Figure 4). Notably, in oxidized MtrF, the ET from heme 2 (–94 mV, Table 1) to FMN (ca. –150 mV (8, 17)) is uphill. (iv) Okamoto et al. demonstrated that binding of FMN at the decaheme cytochrome leads to significant enhancement of ET (8). The present finding, i.e., activating the ET pathways [hemes 9 → 8 → 6 → 1 → 2 → FMN] and [hemes 9 → 8 → 6 → 7→ FMN], fits well with involvement of bound FMN as an electron acceptor (3, 7, 8) when MtrF is reduced, in terms of both the E_m values and the location of the ET pathway (Figure 4). MtrF may alter its function by switching the ET pathway in response to the redox environment. Among the 10 hemes, heme 3, which is surrounded by heme 1, heme2, and heme 4, shows the largest change in E_m, –199 mV, in the transition from oxidized MtrF to reduced MtrF (Table 1); this results in a less uphill, more isoenergetic ET pathway toward heme 5 (Figure 4). Intriguingly, heme 5 has been proposed to serve as a site that can directly reduce extracellular, insoluble substrates (5, 6). Figure 4 shows that the ET pathway [hemes 9 → 8 → 6 → 1 → 3 → 4 → 5] may be more pronounced in reduced MtrF than in oxidized MtrF.

"E_m" reported by Breuer et al.

a) Electrostatic influence of residues. The present results indicate that the ET pathways are downhill along domains IV and II for both reduced and oxidized MtrF. This is due to the different contributions of the electrostatic influences of domains II and IV to the hemes (e.g., Tables 2 and 3). The influence of the protein dielectric volume (see SI for discussion) on E_m, which decreases the solvation of the heme group and lowers the E_m value, is similar in the domain (II, IV) heme pairs, e.g., (heme 4, heme 9) and (heme 3, heme 8) (Table 1). This suggests that the electrostatic influence of residues is the main factor that differentiates the E_m values of hemes in domain II and domain IV.

In the results reported by Breuer et al. (11), residues make unusually large contributions to E_m values, e.g., Asp228 decreased the E_m for heme 2 by –2280 mV, whereas it decreased the E_m for heme 2 by −61

mV in the present study (Table S2). Breuer et al. also listed a number of residues that contributed more than 1000 mV to the E_m shift, which suggests that their E_m values for hemes were determined using overestimated electrostatic influences.

b) E_m values obtained using a TI approach. Breuer et al. calculated E_m values using a TI approach based on MD simulations. To understand how they determined the E_m of MtrF, we also calculated the E_m using a TI approach, based on the original MtrF crystal structure (not the refined MtrF structure). Notably, our total time for sampling simulation is comparable to that reported by Breuer et al. (see Methods). The E_m profiles obtained after equilibrating for 100 ns and 1 μs differ significantly from those reported by Breuer et al. (Figure 5). In addition, the different E_m profiles obtained after equilibrating for 100 ns and 1 μs indicate that the MtrF structure can change even after equilibrating for 100 ns. Breuer et al. equilibrated for only 5 ns (11). The three different E_m profiles obtained using the three different equilibration times indicates that the TI approach is not applicable under the conditions used in the previous studies, and either the equilibrating or sampling simulation times must be insufficient. The difficulty in reproducing the E_m profiles reported in the previous studies, even with a longer equilibration time, argues against the quality of their calculated E_m values.

Breuer et al. seem to have used the original atomic coordinates of the MtrF crystal structure, in which domain I contains marked structural disorder (Figure 2), due to the orientation of the side chains that prevents formation of the β-strands (Figures 1–3). This may be crucial when calculating the E_m using an MD-based TI approach, and may contribute to the uncertainty of their calculated E_m values.

Breuer et al. fixed the protonation states of the heme propionic groups as permanently ionized, even in the presence of reduced heme groups (11). Fixation of the protonation states of titratable residues can also be a fundamental problem when using an MD-based TI approach to calculate the E_m value, in particular for heme proteins, because the protein structure changes with respect to the original atomic coordinates of the crystal structure, to reproduce the initially considered single protonation pattern of the titratable residues. In addition, the protonation state of the heme propionic group is strongly coupled with the redox state of the heme ring, and affects the E_m value, which can often explain the pH-

dependence of the E_m for heme (18-20). Fixation of the protonation states of the heme propionic groups (to be ionized) should also overstabilize the oxidized state of heme and lower the E_m values. Thus, for E_m calculations of heme proteins, it is prerequisite to reproduce the Henderson-Hasselbalch curve for titratable residues near the heme ring (21). This can be achieved only when the partial protonation state of the heme propionic groups is appropriately considered, as demonstrated in a number of electrostatic approaches (e.g., (18-20)).

CONCLUSIONS

The E_m values for the 10 hemes in MtrF were calculated, by solving the linear Poisson-Boltzmann equation and considering the protonation states of all titratable residues and heme propionic groups. The E_m profiles calculated show that the ET pathway proceeds downhill from domain IV to domain II. When MtrF is reduced, the direction of the ET pathway switches, and FMN becomes the terminal electron acceptor. The present findings, i.e., switching of the ET pathways to [hemes 9 → 8 → 6 → 1 → 2 → FMN] or [hemes 9 → 8 → 6 → 7→ FMN], is concordant with FMN acting as the bound electron acceptor (3, 7, 8) when MtrF is reduced.

COMPUTATIONAL PROCEDURES

Initial coordinates and atomic partial charges. The atomic coordinates of MtrF were taken from the X-ray structure of MtrF of *Shewanella oneidensis* at a resolution of 3.2 Å (PDB code, 3PMQ) (5). H atoms were generated and energetically optimized with CHARMM36 (23), and all titratable groups were kept in their standard protonation states. Atomic partial charges of the amino acids were adopted from the all-atom CHARMM36 (23) parameter set. The atomic charges of the low-spin *c*-type heme, including histidine and cysteine ligands, were determined by fitting the electrostatic potential in the neighborhood of these molecules by using the RESP procedure (24). The electronic wave functions were calculated after geometry optimization with the unrestricted DFT method with the B3LYP functional and LACVP* basis sets with the JAGUAR program (25).

Homology modeling of domain I in MtrF. To understand the absence and presence of the β-barrel structure in domain I of the MtrF (5) and MtrC (3) crystal structures, respectively (Figure 2), we prepared the atomic coordinates where the domain I region in the MtrF crystal structure was reconstructed, using a homology modeling approach with the SWISS-MODEL web interface (26). The crystal structure of MtrC from *Shewanella oneidensis* at a resolution of 1.8 Å (PDB code: 4LM8) was used as the main structural template of domain I for reconstruction of MtrF (Figure S1), (3), except for the Lys86 and Lys87 region of MtrC, because the two positively charged residues, "KK," in MtrC are replaced with the single non-polar residue "I−" in MtrF and "G−" in OmcA (Figure S6). The crystal structure of OmcA at a resolution of 2.7 Å (PDB code: 4LMH) (4) was used as the main structural template for the corresponding region. The atomic coordinates obtained were used as the initial structure for subsequent MD simulations (see Results).

MD simulations. MD simulations were performed for the following two purposes: (1) *for E_m calculations using the linear Poisson-Boltzmann approach*, i.e., equilibrating the reconstructed homology model (see above) and obtaining the refined MtrF structure (see Results); (2) *for E_m calculations using a TI approach*, i.e., equilibrating the unmodified, original MtrF crystal structure, as used by Breuer et al. (11) and calculating the E_m values using a TI approach). In both cases, the following procedures were used: for comparison, the protonation states of the titratable residues were identical to those used by Breuer et al. (11); the 10 hemes were oxidized, the acidic groups, including the heme propionic groups, were negatively charged, the basic residues were positively charged, and the histidine residues (except His451) were treated as electrostatically neutral; His451 was positively charged. The respective models were processed as follows: (1) model arrangement in a periodic boundary box, (2) solvation with TIP3P water models (27), (3) structural optimization with positional restraints on heavy atoms, using the initial structure as a reference, (4) MD simulation for 1.0 ns with positional restraints with the Berendsen thermostat at 300 K and the barostat at 1.0 bar (28), (5) MD

simulation with gradual releasing-restraints over 1.0 ns under identical thermostat and barostat conditions, (6) production MD run over 1.0 μs with the Nose-Hoover thermostat (29, 30) at 300 K, with t_t = 0.5 ps, and the Parrinnello-Rahman barostat (31) at 1.0 bar, with t_p = 5.0 ps. All MD simulations above were conducted with an MD engine, GROMACS 5.0.7 (32-34) with an adopted CHARMM36 forcefield (35).

QM/MM calculations. We used the Qsite (36) program code. We employed the unrestricted DFT method with the B3LYP functional and LACVP* basis sets.

***E*$_m$ calculation I: solving the linear Poisson-Boltzmann equation.** To obtain the absolute E_m values for the protein, we calculated the electrostatic energy difference between the two redox states in a reference model system by solving the linear Poisson-Boltzmann equation with the MEAD program (37) and using a known experimentally measured E_m value for bis-histidine ligated heme (−220 mV in water (12)). The difference in the E_m value of the protein relative to the reference system was added to the known E_m value. The ensemble of the protonation patterns was sampled by Monte Carlo method with Karlsberg (38). The linear Poisson-Boltzmann equation was solved using a three-step grid-focusing procedure at resolutions of 2.5 Å, 1.0 Å, and 0.3 Å. Monte Carlo sampling yielded the probabilities [A_{ox}] and [A_{red}] of the two redox states of molecule A. The E_m was evaluated using the Nernst equation. A bias potential was applied to obtain an equal amount of both redox states ([A_{ox}] = [A_{red}]), thereby yielding the redox midpoint potential as the resulting bias potential. To facilitate direct comparisons with previous computational results (20, 39, 40), identical computational conditions and parameters were used; all computations were performed at 300 K, pH 7.0, and an ionic strength of 100 mM (see ref. (41) for the influence of the ionic strength on the calculated E_m values); the dielectric constants were set to e_p = 4 inside the protein and e_w = 80 for water. The size of the ε_P value depends on what is not included explicitly in the protein model used. Lower ε_p values (e.g., ε_p = 1) may be used when all factors that describe electrostatic interactions (e.g., flexibility of the protein structure and

flexibility of the protonation states of the protein titratable residues) are considered explicitly (42, 43). Because we have consistently used $\varepsilon_p = 4$ and $\varepsilon_w = 80$ and reproduced the experimentally measured E_m and pK_a values in many redox active proteins (e.g., heme (20) and flavin (39, 40)), $\varepsilon_p = 4$ seems to be optimal in our computational models.

***E*m calculation II: using TI.** We also calculated the E_m using a TI approach, as used by Breuer et al. (11) (see SI for equations). After restraint-releasing simulations, the initial structures for TI simulations were obtained after equilibration for (i) 0 ns, (ii) 100 ns, and (iii) 1 μs. TI simulations were conducted over 10 ns with an MD time step of 2.0 fs, namely $\Delta\lambda = 2.0e^{-7}$, by reducing a focusing oxidized heme and fixing the protonation states of the other titratable groups. In the present simulations, oxidized heme (Fe^{3+}) was gradually reduced (to Fe^{2+}) over 10 ns, which is comparable to the previous study by Breuer et al. (11). The total sampling simulation time used in the two studies is comparable.

ACKNOWLEDGMENT We thank Hideki Sudo for providing QM/MM-optimized geometries of the heme regions. This research was supported by Grant-in-Aid for JSPS Fellows (to H.C.W.), JST CREST (to H.I.), JSPS KAKENHI (15H00864, 16H06560, 26105012, and 26711008 to H.I.), Materials Integration for engineering polymers of Cross-ministerial Strategic Innovation Promotion Program (SIP to H.I.), and Interdisciplinary Computational Science Program in CCS, University of Tsukuba. Theoretical calculations were partly performed using Research Center for Computational Science, Okazaki, Japan.

REFERENCES

1. Nealson KH & Saffarini D (1994) Iron and manganese in anaerobic respiration: environmental significance, physiology, and regulation. *Annual review of microbiology* 48:311-343.
2. Richardson DJ*, et al.* (2012) The 'porin-cytochrome' model for microbe-to-mineral electron transfer. *Molecular microbiology* 85(2):201-212.
3. Edwards MJ*, et al.* (2015) Redox Linked Flavin Sites in Extracellular Decaheme Proteins Involved in Microbe-Mineral Electron Transfer. *Scientific reports* 5:11677.

4. Edwards MJ*, et al.* (2014) The X-ray crystal structure of Shewanella oneidensis OmcA reveals new insight at the microbe-mineral interface. *FEBS Lett* 588(10):1886-1890.
5. Clarke TA*, et al.* (2011) Structure of a bacterial cell surface decaheme electron conduit. *Proc Natl Acad Sci U S A* 108(23):9384-9389.
6. Brutinel ED & Gralnick JA (2012) Shuttling happens: soluble flavin mediators of extracellular electron transfer in Shewanella. *Applied microbiology and biotechnology* 93(1):41-48.
7. Xu S, Jangir Y, & El-Naggar MY (2016) Disentangling the roles of free and cytochrome-bound flavins in extracellular electron transport from Shewanella oneidensis MR-1. *Electrochim Acta* 198:49-55.
8. Okamoto A, Hashimoto K, Nealson KH, & Nakamura R (2013) Rate enhancement of bacterial extracellular electron transport involves bound flavin semiquinones. *Proc Natl Acad Sci U S A* 110(19):7856-7861.
9. Nakamura R, Ishii K, & Hashimoto K (2009) Electronic absorption spectra and redox properties of C type cytochromes in living microbes. *Angew Chem Int Ed Engl* 48(9):1606-1608.
10. Saito J, Hashimoto K, & Okamoto A (2016) Flavin as an Indicator of the Rate-Limiting Factor for Microbial Current Production in Shewanella oneidensis MR-1. *Electrochim Acta* 216:261-265.
11. Breuer M, Zarzycki P, Blumberger J, & Rosso KM (2012) Thermodynamics of electron flow in the bacterial deca-heme cytochrome MtrF. *Journal of the American Chemical Society* 134(24):9868-9871.
12. Wilson GS (1983) Electrochemical studies of porphyrin redox reactions as cytochrome models. *Bioelectrochem. Bioenerg.* 1:172-179.
13. Hartshorne RS*, et al.* (2009) Characterization of an electron conduit between bacteria and the extracellular environment. *Proc Natl Acad Sci U S A* 106(52):22169-22174.
14. Higgins DG, Thompson JD, & Gibson TJ (1996) Using CLUSTAL for multiple sequence alignments. *Methods Enzymol.* 266:383-402.
15. Ross DE, Flynn JM, Baron DB, Gralnick JA, & Bond DR (2011) Towards electrosynthesis in shewanella: energetics of reversing the mtr pathway for reductive metabolism. *PloS one* 6(2):e16649.
16. Chen IP, Mathis P, Koepke J, & Michel H (2000) Uphill electron transfer in the tetraheme cytochrome subunit of the Rhodopseudomonas viridis photosynthetic reaction center: evidence from site-directed mutagenesis. *Biochemistry* 39(13):3592-3602.
17. Okamoto A*, et al.* (2014) Cell-secreted flavins bound to membrane cytochromes dictate electron transfer reactions to surfaces with diverse charge and pH. *Scientific reports* 4:5628.
18. Voigt P & Knapp EW (2003) Tuning heme redox potentials in the cytochrome c subunit of photosynthetic reaction centers. *J. Biol. Chem.* 278:51993-52001.
19. Mao J, Hauser K, & Gunner MR (2003) How cytochromes with different folds control heme redox potentials. *Biochemistry* 42:9829-9840.
20. Ishikita H & Knapp E-W (2005) Redox potential of cytochrome c550 in the cyanobacterium *Thermosynechococcus elongatus*. *FEBS Lett.* 579:3190-3194.
21. Ullmann GM & Knapp E-W (1999) Electrostatic models for computing protonation and redox equilibria in proteins. *Eur. Bophys. J.* 28:533-551.
22. Breuer M, Rosso KM, & Blumberger J (2014) Electron flow in multiheme bacterial cytochromes is a balancing act between heme electronic interaction and redox potentials. *Proc Natl Acad Sci U S A* 111(2):611-616.
23. Brooks BR*, et al.* (1983) CHARMM: a program for macromolecular energy minimization and dynamics calculations. *J. Comput. Chem.* 4(2):187-217.
24. Bayly CI, Cieplak P, Cornell WD, & Kollman PA (1993) A well-behaved electrostatic potential based method using charge restraints for deriving atomic charges: the RESP model. *J. Phys. Chem.* 97:10269-10280.
25. Jaguar, version 7.5, Schrödinger, LLC, New York, NY, 2008.

26. Kiefer F, Arnold K, Kunzli M, Bordoli L, & Schwede T (2009) The SWISS-MODEL Repository and associated resources. *Nucleic acids research* 37(Database issue):D387-392.
27. Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, & Klein ML (1983) Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* 79:926-935.
28. Berendsen HJC, Postma JPM, Vangunsteren WF, Dinola A, & Haak JR (1984) Molecular-Dynamics with Coupling to an External Bath. *Journal of Chemical Physics* 81(8):3684-3690.
29. Nose S (1984) A Unified Formulation of the Constant Temperature Molecular-Dynamics Methods. *Journal of Chemical Physics* 81(1):511-519.
30. Hoover WG & Holian BL (1996) Kinetic moments method for the canonical ensemble distribution. *Phys Lett A* 211(5):253-257.
31. Parrinello M & Rahman A (1980) Crystal-Structure and Pair Potentials - a Molecular-Dynamics Study. *Phys Rev Lett* 45(14):1196-1199.
32. Bjelkmar P, Larsson P, Cuendet MA, Hess B, & Lindahl E (2010) Implementation of the CHARMM Force Field in GROMACS: Analysis of Protein Stability Effects from Correction Maps, Virtual Interaction Sites, and Water Models. *Journal of chemical theory and computation* 6(2):459-466.
33. Pronk S*, et al.* (2013) GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. *Bioinformatics* 29(7):845-854.
34. Abraham MJ*, et al.* (2015) GROMACS: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX* 1–2:19-25.
35. Best RB*, et al.* (2012) Optimization of the additive CHARMM all-atom protein force field targeting improved sampling of the backbone phi, psi and side-chain chi(1) and chi(2) dihedral angles. *Journal of chemical theory and computation* 8(9):3257-3273.
36. QSite, version 5.8, Schrödinger, LLC, New York, NY, 2012.
37. Bashford D & Karplus M (1990) Pkas of Ionizable Groups in Proteins - Atomic Detail from a Continuum Electrostatic Model. *Biochemistry* 29(44):10219-10225.
38. Rabenstein B & Knapp EW (2001) Calculated pH-dependent population and protonation of carbon-monoxy-myoglobin conformers. *Biophys. J.* 80(3):1141-1150.
39. Ishikita H (2007) Contributions of protein environment to redox potentials of quinones in flavodoxins from *Clostridium beijerinckii*. *J. Biol. Chem.* 282:25240-25246.
40. Ishikita H, Eger BT, Okamoto K, Nishino T, & Pai EF (2012) Protein conformational gating of enzymatic activity in xanthine oxidoreductase. *Journal of the American Chemical Society* 134:999-1009.
41. Ishikita H & Knapp E-W (2005) Oxidation of the non-heme iron complex in photosystem II. *Biochemistry* 44:14772-14783.
42. Schutz CN & Warshel A (2001) What are the dielectric constants of proteins and how to validate electrostatic models? *Proteins* 44:400-417.
43. Warshel A, Sharma PK, Kato M, & Parson WW (2006) Modeling electrostatic effects in proteins. *Biochim. Biophys. Acta* 1764:1647-1676.

Figure legends

Figure 1. Overview of multi-heme cytochromes. (a) The crystal structure of MtrF (5). Hemes 2 and 7 have been proposed to be located near the FMN binding site (5, 6). The orientation of MtrF with respect

to the outer membrane was proposed by Clarke et al. (5). α-helixes and β-strands are depicted as purple or red coils and yellow ribbons, respectively. (b) The crystal structure of MtrC (3).

Figure 2. Calculated B-factors of the backbone Cα atoms in domain I (residues 44–186), corresponding to the original MtrF crystal structure (black dotted line) or the (domain I replaced) refined structure (red solid line). For comparison, the B-factors stated in the PDB file (PDB ID: 3PMQ) are also shown (black dots).

Figure 3. Orientation of the side-chains in domain I of MtrF. (a) The original MtrF crystal structure (PDB ID: 3MPQ), in which charged and polar residues are oriented toward the inner core and hydrophobic residues are exposed to the bulk (5). (b) The refined structure used for E_m calculations (solving the linear Poisson-Boltzmann equation).

Figure 4. (a) E_m profiles of oxidized (red) and reduced (blue) MtrF. See Table 1 for the E_m values. Black dotted lines indicate the E_m for bound FMN (8, 17). (b) Geometry of the ET pathways for oxidized (left panel) and reduced (right panel) MtrF. Thick solid arrows indicate the main downhill pathways. [hemes 9 → 8 → 6 → 1 → 3 → 4 → 5] may be more pronounced in reduced MtrF than in oxidized MtrF (dotted arrows).

Figure 5. E_m profiles obtained for oxidized MtrF, using a TI approach based on MD simulations, in which the initial structures were obtained after equilibrating for 0 ns (black), 100 ns (pink), and 1 μs (orange). It should be noted that Breuer et al. uniformly added the constant $C = -1567$ mV (11) to

reproduce the E_m range of −44 to −312 mV (5) reported for the hemes in MtrF. The corresponding constants were 453 mV, 434 mV, and 511 mV after equilibrating for 0 ns, 100 ns, and 1 μs, respectively.