Electron transfer pathways in a multiheme cytochrome MtrF

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Edited by Arieh Warshel, University of Southern California, Los Angeles, CA, and approved February 2, 2017 (received for review October 25, 2016)

In MtrF, an outer-membrane multiheme cytochrome, the 10 heme groups are arranged in heme binding domains II and IV along the pseudo-C2 axis, forming the electron transfer (ET) pathways. Previous reports based on molecular dynamics simulations showed that the redox potential (Em) values for the heme pairs located in symmetrical positions in domains II and IV were similar, forming bidirectional ET pathways [Breuer M, Zarzycki P, Blumberger J, Rosso KM (2012) J Am Chem Soc 134(24):9868–9871]. Here, we present the Em 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 Em values indicated that the ET is more likely to be downhill from domain IV to II because of localization of acidic residues in domain IV. Reduction of hemes in MtrF lowered the Em values, resulting in switching to alternative downhill ET pathways that extended to the flavin binding sites. These findings present an explanation of how MtrF serves as an electron donor to extracellular substrates.

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 use 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, multiheme 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 transmembrane region (MtrB), and a multiheme cytochrome on the outer membrane (MtrC) (2)]. The 1.8 Å resolution crystal structure of MtrC shows that domains I and III are both β-barrel domains and structurally similar (Fig. 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 homolog 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-strands, whereas domain III is a β-barrel domain (Fig. 1) (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 (Fig. 1). Using the protein–protein interface server, Clarke et al. (5) proposed that heme 10 is on the periplasmic side and that heme 5 is solvent-exposed. They also proposed that the two ET pathways that are terminated by heme 2 or 7 may function in the 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 with 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 because of continuously supplied electrons (9). In the extracellular ET (10). To understand the mechanism of ET in MtrF, the redox potential (Em) values of the 10 heme groups must be determined. Although protein film voltammetry showed that the Em 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. (11) calculated the Em values using a thermodynamic integration (TI) approach based on molecular dynamics (MD) simulations. Breuer et al. (11) uniformly added the constant C = --1,567 mV to reproduce the Em range from --44 to --312 mV (5) reported for the hemes in MtrF by protein film voltammetry. Hemes 1 (--41 mV) and 6 (--51 mV) in the middle of the ET chain had the highest Em values, whereas hemes 4 (--266 mV) and 9 (--279 mV) had the lowest Em 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) (Fig. S1).

Here, we present the Em 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 nonpolar and polar residues are likely to alternate along the β-strands, with the nonpolar residues oriented inward, forming the hydrophobic core, whereas the polar residues are exposed to the bulk solvent (Fig. S2). Indeed, domain I of MtrF shows the alternating polar/nonpolar 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 protein interior (e.g., Asp65, Asn88, Arg150, Lys154, Asp174, and Gln176) (see Fig. 3.4 and Fig. S1) (5). The MtrF crystal structure (5) shows few interstrand 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 (Fig. 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 nonpolar residues and the location of the interstrand H bond in domain I of MtrF (Fig. S1); (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). 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 (Fig. 2) and that the β-barrel structure of domain I was stable (Fig. 3B and Fig. 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 (Fig. 1B). Thus, we replaced domain I of the MtrF crystal structure with the one obtained by 1.0-μs MD simulation and used the structure for the following quantum mechanical/molecular mechanical (QM/MM) calculations.

In the MtrF crystal structure, His ligands [e.g., hemes 6 and 10 (Fig. 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 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 (atomic coordinates are in Dataset S1).

**$E_m$ Values.** Breuer et al. (11) calculated the $E_m$ for hemes in oxidized MtrF, where $E_m$ for the focusing heme was obtained in the presence of the other nine hemes being in the oxidized states (i.e., oxidized MtrF). In this study, we calculated the $E_m$ values 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 on 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 [−44 to −312 mV (5)] or that reported for the MtrCAB complex [0 to −450 mV (12)].

**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 II (Fig. 4). In particular, among the heme pairs in domains II and IV, which are located at symmetrical positions with respect to the pseudo-C2 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 hemes 9 and 8 in domain IV with respect to hemes 3 and 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. (11) also reported that Asp631 decreased the $E_m$ for heme 9, the contribution was $-1,362$ mV, 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 (Fig. S5). This result suggests that the ET pathways could still be downhill, even if the orientation of Asp631 was 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) (15) (Fig. S1). Thus, each symmetrical pair of hemes is more likely to have different \( E_m \) values (Table 1) because of the contributions of different types of residues as shown in Tables 2 and 3.

MtrF, MtrD, and MtrE are homologs of MtrC, MtrA, and MtrB, respectively. In the MtrCAB complex, MtrC has been reported to have higher \( E_m \) values than MtrA based on electrophoretical analysis using cyclic voltammetry (12) [i.e., the ET pathway from MtrA to MtrC is downhill, whereas the ET pathway (MtrC pathway) can also mediate reversible ET (14)]. 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 in the uphill ET pathway [e.g., the cytochrome c subunit of photosynthetic reaction centers from Blastochloris viridis (15)]; this fact also suggests that a completely symmetric \( E_m \) profile (11) is not necessarily required to facilitate the reversible ET (14) 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] (Fig. 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] (Fig. 4).

Intriguingly, (i) in reduced MtrC, FMN showed pronounced binding affinity compared with 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 bound FMN is not known for MtrF, but for MtrC, it is reported to be \(-150\) mV using differential pulse voltammetry (8, 16). If this \( E_m \) value 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 (Fig. 4). Notably, in oxidized MtrF, the ET from heme 2 (\(-94\) mV (Table 1)) to FMN (\(-150\) mV (8, 16)) is uphill. (iv) Okamoto et al. (8) showed that binding of FMN at the decapheme cytochrome leads to significant enhancement of ET. This 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 (Fig. 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 hemes 1, 2, and 4, shows the largest change in \( E_m \) (176 mV), in the transition from oxidized MtrF to reduced MtrF (Table 1); this \( E_m \) shift results in a less uphill, more isoeneryonic ET pathway toward heme 5 (Fig. 4). Intriguingly, heme 5 has been proposed to serve as a site that can directly reduce extracellular insoluble substrates (5, 6). Fig. 4 shows that the ET pathway [hemes 9 → 8 → 6 → 1 → 3] may be more pronounced in reduced MtrF than in oxidized MtrF.

#### \( E_m \) Reported by Breuer et al.

**Electrostatic influence of residues.** In the present study, the ET pathways are downhill along domains IV and II for both reduced and oxidized MtrF, which is caused by the different contributions of the electrostatic influences of domains II and IV to the hemes (Tables 2 and 3). The influence of the protein dielectric volume (SI 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). Thus, the electrostatic influence of residues is the main factor that differentiates the \( E_m \) values of hemes in domains II and 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 \(-2,280\) mV), whereas it decreased the \( E_m \) for heme 2 by \(-61\) mV in this study (Table S2). Breuer et al. (11) also listed a number of residues that contributed more than 1,000 mV to the \( E_m \) shift, which suggests that their \( E_m \) values for hemes were determined using overestimated electrostatic influences.

#### \( E_m \) values obtained using a TI approach

Breuer et al. (11) 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 with that reported by Breuer et al. (11) (Computational Procedures). The \( E_m \) profiles obtained after equilibrating for 100 ns and 1 µs differ

### Table 1. Calculated \( E_m \) values and factors that determine the \( E_m \) values for oxidized and reduced MtrF (in millivolts)

<table>
<thead>
<tr>
<th>Domain</th>
<th>Heme</th>
<th>Oxidized</th>
<th>Reduced</th>
<th>Contribution to ( E_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[Protein volume]</td>
<td>[Charge]</td>
<td></td>
</tr>
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<td></td>
<td></td>
<td>Oxidized</td>
<td>Reduced</td>
<td>Oxidized</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>-178</td>
<td>-252</td>
<td>60</td>
</tr>
<tr>
<td>II</td>
<td>4</td>
<td>-141</td>
<td>-290</td>
<td>51</td>
</tr>
<tr>
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</tr>
<tr>
<td>IV</td>
<td>9</td>
<td>-338</td>
<td>-392</td>
<td>34</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>-296</td>
<td>-277</td>
<td>30</td>
</tr>
</tbody>
</table>

*Contribution of the protein (dielectric) volume corresponds to the \( E_m \) shift caused by loss of solvation of heme in the protein environment.*
significantly from those reported by Breuer et al. (11) (Fig. 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. (11) equilibrated for only 5 ns. The three different $E_m$ profiles obtained using 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. (11) seem to have used the original atomic coordinates of the MtrF crystal structure, in which domain I contains marked structural disorder (Fig. 2) because of the orientation of the side chains that prevents formation of the β-strands (Figs. 1, 2, and 3). Using the unstable structure 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. (11) fixed the protonation states of the heme propionic groups as permanently ionized, even in the presence of reduced heme groups. 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 (Table S3) and affects the $E_m$ value, which can often explain the pH dependence of the $E_m$ for heme (17–19). 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 a prerequisite to reproduce the Henderson–Hasselbalch curve for titratable residues in the vicinity of the heme ring (20), which can be achieved only when the partial protonation state of the heme propionic groups is appropriately considered as shown in a number of electrostatic approaches (17–19).

### 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 II. When MtrF is reduced, the direction of the ET pathway switches, and FMN becomes the terminal electron acceptor. These findings (i.e., switching of the ET pathways to hemes 9 → 8 → 6 → 1 → 2 → 10) may be more pronounced in reduced MtrF than in oxidized MtrF (dotted arrows).

<table>
<thead>
<tr>
<th>Residue pairs</th>
<th>Contribution to $E_m$ (heme 4) – $E_m$ (heme 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp306 Asp631</td>
<td>124</td>
</tr>
<tr>
<td>Ser247 Gly544</td>
<td>29</td>
</tr>
<tr>
<td>Ile229 Asp518</td>
<td>28</td>
</tr>
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</table>

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**Fig. 4.** (A) $E_m$ profiles of oxidized (red) and reduced (blue) MtrF. Table 1 shows the $E_m$ values. Black dotted lines indicate the $E_m$ values for bound FMN (8, 16). (B) Geometry of the ET pathways for (Left) oxidized and (Right) reduced MtrF. Thick solid arrows indicate the main downhill pathways; [hemes 9 → 8 → 6 → 1 → 3] may be more pronounced in reduced MtrF than in oxidized MtrF (dotted arrows).
profiles obtained for oxidized MtrF using a TI approach based on the range of Watanabe et al. in MtrF and G was evaluated using the calculations using a TI approach after geometry optimization. The value of the E value for bis-histidine ligated heme values for the protein, we calculated the at a resolution value depends on what is not –

Domain II Domain IV Contribution to E@GeneratedValue from (heme 3) – EGeneratedValue from (heme 8)

Arg262 Ile557 88
Ile229 Asp518 74
Asn264 Ser558 46
Trp306 Asp631 43
Gln210 Asp490 28

The residue pair Glu102 (Domain I)/Asp377 (Domain IV) also contributes to an increase of 68 mV in EGeneratedValue from (heme 3) – EGeneratedValue from (heme 8).

QM/MM Calculations. We used the Qsite (34) program code. We used 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 (35) and using a known experimentally measured E_m value for bis-histidine ligated heme [−220 mV in water (36)]. 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 (37). The linear Poisson–Boltzmann equation was solved using a three-step grid-focusing procedure at resolutions of 2.5, 1.0, and 0.3 Å. Monte Carlo sampling yielded the probabilities [Aox] and [Arred] 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 ([Aox] = [Arred]), thereby yielding the redox midpoint potential as the resulting bias potential. To facilitate direct comparisons with previous computational results (19, 38, 39), identical computational conditions and parameters were used. All computations were performed at 300 K, pH 7.0, and an ionic strength of 100 mM (ref. 40 discusses the influence of the ionic strength on the calculated E_m values); the dielectric constants were set to 4 for the protein interior (εp) and 80 for water (εw). The size of the εp value depends on what is not positively charged. The respective models were processed as follows: (i) model arrangement in a periodic boundary box, (ii) solvation with TIP3P water models (25), (iii) structural optimization with positional restraints on heavy atoms using the initial structure as a reference, (iv) MD simulation for 1.0 ns with positional restraints with the Berendsen thermostat at 300 K and the barostat at 1.0 bar (26), (v) MD simulation with gradual releasing restraints over 1.0 ns under identical thermostat and barostat conditions, and (vi) production MD run over 1.0 μs with the Nose–Hoover thermostat (27, 28) at 300 K with τb = 0.5 ps and the Parrinello–Rahman barostat (29) at 1.0 bar with τp = 5.0 ps. All MD simulations above were conducted with an MD engine GROMACS 5.0.7 (30–32) with an adopted CHARMM36 force field (33).

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 (Fig. 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 (24). The crystal structure of MtrC from Shewanella oneidensis at a resolution of 1.8 Å (PDB ID code 4LM8) was used as the main structural template of domain I for reconstruction of MtrF (Fig. 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 nonpolar residue I– in MtrF and G– in OmcA (Fig. S6). The crystal structure of OmcA at a resolution of 2.7 Å (PDB ID 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 (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 (Results)] (2) and 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: (i) model arrangement in a periodic boundary box, (ii) solvation with TIP3P water models (25), (iii) structural optimization with positional restraints on heavy atoms using the initial structure as a reference, (iv) MD simulation for 1.0 ns with positional restraints with the Berendsen thermostat at 300 K and the barostat at 1.0 bar (26), (v) MD simulation with gradual releasing restraints over 1.0 ns under identical thermostat and barostat conditions, and (vi) production MD run over 1.0 μs with the Nose–Hoover thermostat (27, 28) at 300 K with τb = 0.5 ps and the Parrinello–Rahman barostat (29) at 1.0 bar with τp = 5.0 ps. All MD simulations above were conducted with an MD engine GROMACS 5.0.7 (30–32) with an adopted CHARMM36 force field (33).
included experimentally measured \( E_m \) and \( pK_a \) values in many redox active proteins [e.g., heme (19) and flavin (38, 39)], \( e_p \) 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). 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 \( \Delta t = 2.0 \times 10^{-15} \), by reducing a focusing oxidized heme and fixing the protonation states of the other titratable groups. In these simulations, oxidized heme (Fe\(^{3+}\)) was gradually reduced (to Fe\(^{2+}\)) over 10 ns. The total sampling simulation time used in the present study by Breuer et al. (11) is comparable with the present study.

**Acknowledgments.** We thank Hideki Sudo for providing QMMM-optimized geometries of the heme regions. Theoretical calculations were partly performed using the Research Center for Computational Science (Okazaki, Japan). This research was supported by a Grant-in-Aid for the Japan Society for the Promotion of Science (JSPS) (Young (H.C.W.), JP26105012 (to H.I.), and JP26711008 (to H.I.)), the Materials Integration for Engineering Polymers of Cross-Ministerial Strategic Innovation Promotion Program (H.I.), and the Interdisciplinary Computational Science Program in Center for Computational Sciences, University of Tsukuba.