# Kinetics of Trifurcated Electron Flow in the Deca-heme Bacterial Proteins MtrC and MtrF

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**The bacterium Shewanella oneidensis has evolved a sophisticated electron transfer machinery to export electrons from the cytosol to extracellular space during extracellular respiration. At the heart of this process are deca-heme proteins of the Mtr pathway, MtrC and MtrF, located at the external face of the outer bacterial membrane. Crystal structures have revealed these proteins bind 10 c-type hemes arranged in the peculiar shape of a staggered cross that trifurcates the electron flow, presumably to reduce extracellular substrates while directing electrons to neighbouring MHCs at either sides along the membrane. Especially intriguing is the design of the heme junctions trifurcating the electron flow: they are made of co-planar and T-shaped heme pair motifs with relatively large and seemingly unfavourable tunneling distances. Here we use electronic structure calculations and molecular simulation to show that the side chains of the heme rings, in particular the cysteine linkages inserting in the space between co-planar and T-shaped heme pairs, strongly enhance electronic coupling in these two motifs. This results in a $\approx 10^3$-fold speed-up of ET steps at heme junctions that would otherwise be rate-limiting. The predicted maximum electron flux through the solvated proteins is remarkably similar for all possible flow directions, suggesting that MtrC and MtrF shuttle electrons with similar efficiency and reversibly in directions parallel and orthogonal to the outer membrane. No major differences in the ET properties of MtrC and MtrF are found implying that the different expression levels of the two proteins during extracellular respiration is not related to redox function.**

extracellular respiration | electron transfer | heme | molecular dynamics | density functional theory

Multi-heme cytochromes (MHC) are expressed by the bacteria *Shewanella oneidensis* and *Geobacter sulfurreducens* to shuttle electrons from the inside of the cell across the periplasm and outer membrane to extracellular space in a process termed extracellular respiration(1). They are part of a fascinating electron export machinery that allows the bacterium to survive at reduced $O_2$ levels by transferring electrons, accumulated by metabolic activity, to electron acceptors *outside* the cell, e.g. transition metal oxide minerals $Fe_2O_3$ and $MnO_2$. The bacteria's ability to electronically wire the cytosol with extracellular space has attracted much interest for their use in the clean-up of water and soil containing radioactive isotopes(2), for mediatorless microbial fuel cells(3, 4) and microbial electrosynthesis(5–7) (see review Ref. (8)). The exquisite electron transfer properties of their MHCs has also sparked much interest for their use in bioelectronic junctions and devices(9–12). It was recently shown that two MHCs from *S. oneidensis*, STC and MtrF, are up to 1000-fold more conductive than other metallo-proteins such as azurin and single-heme cytochromes(12), which might open up a host of new electronic applications at the biotic/abiotic interface.

Crystal structures of several MHCs have been resolved in recent years(13–17); the structures of some of the most prominent MHCs of *S. oneidensis* are shown in fig. 1. Among the largest, the deca-heme proteins MtrC(17) and MtrF(15) (panels A and B) arrange ten tightly packed bis-His coordinated c-type hemes in the peculiar shape of a staggered cross: a vertically aligned octa-heme chain is intersected horizontally by a tetra-heme chain. Located on the external surface of the outer membrane, MtrC (MtrF) is part of the MtrCAB (MtrFDE) complex that spans the outer membrane (see panel E) and transmits electrons over distances larger than 100 Å. Electron input from the electron donor MtrA (MtrD) occurs at one of the termini of the octa-heme chain, speculated to be heme 10(15). Subsequent electron flow through MtrC may occur in three different directions, along the octa-heme chain to heme 5, or towards the side exits of the tetra-heme chain, hemes 2 and 7.

While there may be multiple reasons for the evolution of cytochromes that feature a staggered heme cross, a clue for a possible functional role came from recent in-vivo(18) and electron cryotomography studies(19). It was shown that the micrometer-long cellular appendages that *S. oneidensis* form upon reduced $O_2$ levels (sometimes referred to as "biological nanowires") are in fact extensions of the outer membrane rather than pilin-based structures(20, 21), with MtrCAB dis-

## Significance Statement

Certain bacteria survive in anoxic environments by switching from aerobic to anaerobic respiration: in place of $O_2$ they reduce diverse substrates *outside* the cell. Multi-heme cytochromes (MHC) spanning the outer membrane have been identified as the essential building blocks for this process. Here we provide molecular-level insight into the electron flow in two deca-heme proteins. Our study reveals that electron hopping through these proteins is strongly enhanced by cysteines at heme junctions that trifurcate the electron flow. We believe this to be a general design principle in MHCs for acceleration of ET steps that would otherwise be too slow for respiration. Our study uncovers a natural design principle of significance to an entire class of proteins involved in biological ET.

X.J., B.B., F.G. and C.B. carried out molecular simulations, Z.F. contributed computer code, X.J., M. B. and J.B. analyzed results and J.B designed the work.

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tributed along their length(18, 19), as schematically indicated in fig. 1E. Adjacent MtrCAB complexes are thought to interact via the tetra-heme chains of MtrC to facilitate micro-meter long electron transfer along the outer membrane as observed by c-AFM(22), while the octaheme-chains support ET away from the membrane and on to extracellular substrates. In this way, the heme cross motif helps supply the surface of the membrane with electrons while reducing extracellular substrates. Yet, the kinetics of the trifurcated electron flow in MtrC remains to be elucidated; does this protein transfer electrons equally well in the direction parallel to the membrane and away from it?

In our previous work we have used quantum chemistry and molecular simulation to obtain a first insight into the workings of solvated MHCs at the single-protein level.(23–25) In an early study, we calculated the reduction potentials for all 10 hemes in all-oxidized (all-ox) MtrF using MD simulation(26), and more recently Barrozo *et al.* reported heme reduction potentials for MtrF and MtrC in the all-ox and all-reduced (all-red) states(27). Both studies agreed that the free energy profile for electron flow along the protein has ups and downs, yet resulting in near thermoneutral ET along the octaheme chain. In terms of kinetics, we found that heme-heme electronic couplings are about three orders of magnitude smaller than reorganization free energy, which implies that intraprotein ET through solvated MHCs occurs via heme-to-heme hopping. However, our calculations fell short of reproducing the ≈ nano-Ampere (nA) currents reported in STM measurements(9, 11). Even after accounting for partial protein hydration in these experiments, the computed STM currents remained underestimated by about two orders of magnitude.

This discrepancy has motivated us to take a closer look at the staggered cross heme motif built into MtrC and MtrF. The trifurcation of the electron flow is established by two junctions in the middle of the protein comprised of T-shaped (8-6, 1-3) and co-planar heme pairs (6-1, 6-7, 1-2). Inspection of the crystal structure reveals relatively large heme-to-heme edge distances in these motifs suggesting that the ET steps across the junctions may limit to overall electron flow through the protein. In this respect, we note that the smaller tetra-heme protein STC (fig. 1 (D)) also features two T-shaped heme pairs, similarly as in MtrF and MtrC, though no co-planar pairs. We found that cysteine linkages, which chemically attach the heme rings to the protein frame, enhance electronic coupling between the T-shaped heme pairs in STC. The effect of the cysteine linkages has not been included in our previous computations on MtrF(28) and calls for a re-calculation of electronic couplings for this protein, especially in the context of the persisting mismatch between computation and experiment for STM currents(11).

Returning to the bacterium *S. oneidensis*, a puzzling observation is that under anoxic conditions only MtrCAB and no MtrDEF is expressed, even though it is known that MtrF can functionally replace MtrC(29, 30). As pointed out by Barrozo *et al.*(27), this apparent redundancy is unusual and the conditions under which the genes for MtrDEF are expressed remain largely unknown. It begs the question whether different expression levels of the two proteins is due to differences in their electron transfer properties. Does MtrC conduct electrons better than MtrF? To answer this and the above questions we present in the current paper all ET parameters, heme-to-heme

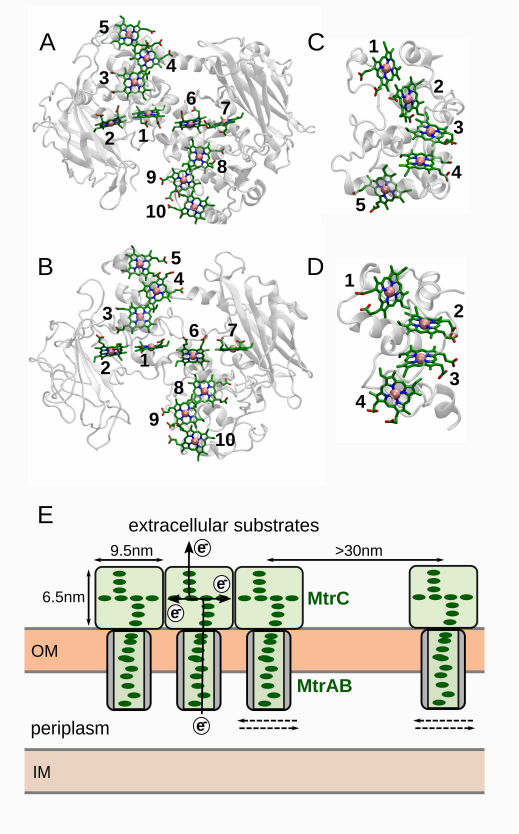


**Fig. 1.** Crystal structures of decaheme cytochromes MtrC, pdb id: 4LM8(17) (A); MtrF, pdb id: 3PMQ(15) (B); penta-heme cytochrome NrfB, pdb id: 2OZY(13) (C) and tetraheme cytochrome STC, pdb id: 1M1Q(31) (D). The bis-His coordinated c-type heme rings are depicted in green, Fe atoms in purple and the protein secondary structures in grey. (E) Cartoon representation of a possible arrangement of MtrCAB complexes in the bacterial outer membrane during extracellular respiration (OM), inspired by the cryotomography study of Ref. (19). Electrons from the periplasm are transferred across the OM via the deca-heme protein complex MtrAB and passed on to the deca-heme protein MtrC where the electron flow is trifurcated in directions parallel and orthogonal to the OM. The spacing between the centers of adjacent MtrC and MtrA molecules is typically about 10 nm, i.e. close contact, but gaps larger than 30 nm were also observed and may be overcome by lateral protein diffusion within the membrane, as indicated by dashed arrows.(19) IM stands for inner bacterial membrane.

ET rate constants and protein-limited electron flux through MtrC, calculated for exactly the same conditions as for MtrF. This undertaking is very timely because the crystal structure of MtrC has recently become available. Whilst Barrozo *et al* have recently presented a preliminary characterization of the ET kinetics for MtrC,(27) though with outdated electronic couplings from MtrF, a full and up-to-date characterization for this protein is outstanding.

## Results

**Heme-heme electronic interaction.** Electronic coupling matrix elements for electron hopping between adjacent $Fe^{2+}/Fe^{3+}$-heme pairs have been calculated along molecular dynamics trajectories for the solvated MtrC and MtrF. The coupling calculations were carried out for two QM models on structures extracted from the MD run, one where the two bis-His hemes are modelled by unsubstituted Fe-porphin rings axially ligated by two N-methyl imidazoles, hereafter referred to as the mini-

mum model, and one where, in addition, all of the side chains of both heme rings are included, hereafter referred to as the large model. Details on the MD simulations and the density functional theory (DFT)-based coupling calculations can be found in *Materials and Methods* and in the SI appendix.

The results are shown in fig. 2, where we have also included previously reported couplings for the small tetra-heme protein STC(32). The data for the minimum model (panel A) show the expected exponential decay with respect to the heme edge-to-heme edge distance $r$, $\langle|H_{ab}|^2\rangle^{1/2} = A\exp[-\beta(r-r_0)/2]$. The scatter around the mean values is due to thermal motion of the heme rings ($T = 300$ K). The couplings decrease in the order stacked > T-shaped > co-planar heme-heme motif. In the stacked motif the hemes approach one another up to van-der-Waals distance (3.5−5 Å) resulting in couplings of several meVs, whereas in the T-shaped and co-planar motifs the edge-to-edge distances are larger (5−8 Å), and the couplings are an order of magnitude smaller, typically a few 0.1 meV or less. The distance decay constant $\beta$ and the prefactor $A$ are determined to be 2.26 Å$^{-1}$ and 3.49 meV, respectively ($R^2 = 0.99$, $r_0 = 3.6$ Å), in good agreement with the ones reported previously for data from MtrF only(28). The thermally averaged couplings for each heme pair of MtrC are depicted in fig. 2B clearly illustrating how the couplings decrease from relatively large values for the stacked motif at electron input and exit sites of the octa-heme chain (hemes 10 and 5), to smaller values for the T-shaped and co-planar motifs in the middle of the protein. Particularly small is the electronic coupling for the co-planar pair 1-6 in the center of the protein due to their relatively large edge-to-edge distance (7.0 Å in the crystal structure(17)).

Yet, the situation is strikingly different when the heme side chains are included in the coupling calculation. While the values for the stacked motif hardly change, they increase significantly for co-planar and T-shaped motifs, respectively, to values that are just slightly below the ones for the stacked motif (fig. 2C). Consequently, all couplings now fall in a rather narrow range of about 0.9-3.5 meV for MtrC (0.7-4.5 meV for MtrF). For further discussion, we define the coupling enhancement as the ratio $r_{dft}^{1/2} = [\langle|H_{ab}^{l}|^2\rangle/\langle|H_{ab}^{m}|^2\rangle]^{1/2}$, where $H_{ab}^{l}$ and $H_{ab}^{m}$ are the coupling matrix element for heme-to-heme electron tunneling for the large (l) and minimum (m) QM models, and $\langle\cdots\rangle$ denotes the thermal average over MD snapshots. We find that most of the coupling enhancement is due to the cysteine linkages that insert in the space between co-planar and T-shaped heme motifs (inset of panel C). In case of the co-planar heme pair 1-6, where the coupling enhancement effect is the greatest ($r_{dft}^{1/2} = 50$ for MtrC (30 for MtrF)), Cys189(197) and Cys499(476) which covalently link hemes 1 and 6 to the protein backbone, approach one another up to a S-S distance of 4.0 (3.8) Å. According to our calculations, the sulfur 3p orbital of Cys189(197) weakly mixes with the Fe-heme frontier orbitals of heme 1, and a similar mixing occurs for Cys499(476) and heme 6. The small delocalization of the frontier orbital over the S atoms leads to a sizable increase in orbital overlap and consequently electronic coupling. Similar albeit smaller coupling enhancements occur for the T-shaped pairs 8-6 ($r_{dft}^{1/2} = 6$ (8)) and 1-3 ($r_{dft}^{1/2} = 3$ (4)), where only one cysteine inserts between the hemes.

A consequence of the mixing of frontier orbital amplitude over heme side chains is that the heme edge-to-heme edge distance is no longer a good distance metric for heme-to-heme electron tunneling. The spread of coupling values around the best fit for exponential distance decay is very large ($R^2 = 0.57$, see SI Appendix, fig. S1). Instead, we use the shortest distance between any heavy atom (C, N, O, S) of the porphin ring plus side chains. Using this metric, all couplings shift to shorter distances and can be fit to an exponential distance decay with parameters similar to the ones for the minimum model in panel (A), $\beta = 2.63$ Å$^{-1}$, $A = 2.57$ meV ($R^2 = 0.97$, fig. 2D), characteristic for through-space tunneling. Yet, the scatter around the mean values is still significantly larger than for the minimum model. Most likely, this is because the degree of delocalization of the frontier orbitals over the side chains varies more strongly with intramolecular heme geometry than for the unsubstituted porphyrine rings and this effect is independent on inter-heme distance.

We would like to emphasize that electronic couplings calculated here are for through-space tunneling between adjacent heme cofactors including all side chains and the Cys link-

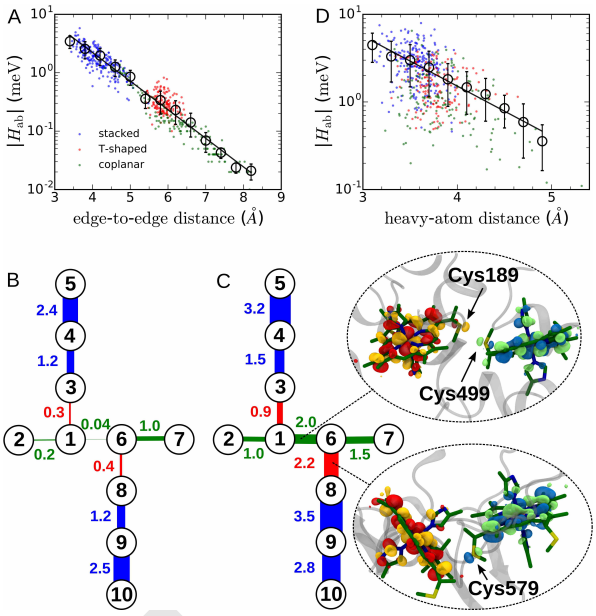


**Fig. 2.** Heme-heme electronic coupling matrix elements, $|H_{ab}|$, in MtrC, MtrF and STC. The distance dependence of electronic couplings is shown in (A) for the minimum (m) QM model ($H_{ab} = H_{ab}^{m}$) comprised of the unsubstituted heme rings plus axial ligands and in (D) for the large (l) QM model ($H_{ab} = H_{ab}^{l}$) where, in addition, all heme side chains are included, in particular the Cys linkages. The couplings are calculated on structures obtained from MD simulation at room temperature. They are color-coded according to the relative orientations of electron donating and accepting hemes: stacked motif in blue (heme pairs 10-9, 9-8, 3-4, 4-5 in MtrC and MtrF and 2-3 in STC), T-shaped in red (8-6 and 1-3 in MtrC and MtrF and 1-2, 3-4 in STC), and co-planar in green (6-1, 6-7 and 1-2 in MtrC and MtrF). Root-mean-square averages of the scattered data points were calculated for bins of width 0.4 Å (A) and 0.2 Å (D) and are denoted by black circles with error bars indicating the root-mean-square fluctuations. Fits to an exponential are indicated by a black line. In (A) the shortest heme edge-to-edge distance is used and in (B) the shortest distance between any heavy atom of heme ring and side chains. Electronic couplings averaged for each adjacent heme pair in MtrC, $\langle|H_{ab}|^2\rangle^{1/2}$, are indicated for the minimum QM model (panel B) and for the large QM model (panel C). The thickness of the bars connecting adjacent hemes is proportional to the average coupling. The insets in (C) depict the enhancement of electronic couplings due to Cys linkages inserting in the space between co-planar heme pair 6-1, and T-shaped heme pair 8-6. One of the three Fe $d(t_{2g})$-heme orbitals on electron donor and acceptor hemes contributing to electronic coupling are drawn as red/yellow and green/blue isosurfaces (denoted $d_i^D$ and $d_j^A$ in SI appendix). Similar coupling enhancements are found for MtrF.

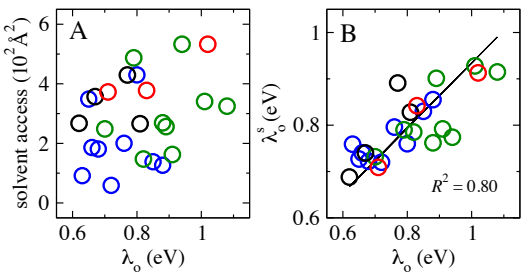


**Fig. 3.** Outer-sphere reorganization free energy, $\lambda_o$, for heme-to-heme ET in MtrC (blue), MtrF (green), NrfB (black) and STC (red), as obtained from MD simulations. Values for MtrC and NrfB taken from current simulations (SI Appendix, Table S1), values for MtrF taken from Ref. (35) and for STC from Ref. (32). Correlations are shown between $\lambda_o$ and (A) the solvent accessible surface area (SA)(36) of corresponding heme pairs, (B) Marcus continuum estimates for outer-sphere reorganization free energy,$\lambda_o^s$ with SA-dependent static dielectric constant (see main text for details).

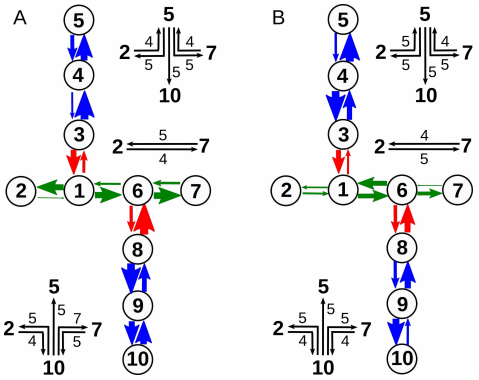


**Fig. 4.** Kinetics of trifurcated electron flow in solvated MtrC (A) and MtrF (B). The thickness of the colored arrows connecting hemes is proportional to the heme-to-heme ET rate constants in the all-ox state, summarized in SI Appendix, Table S1. See fig. 1 for heme numbering. Insets show the possible flow directions between the terminal hemes 10, 5, 7, 2 with the logarithm of the maximum, protein-limited steady-state electron flux, $\log_{10}(J_{\max}/s^{-1})$, indicated for each flow direction. The electron flux is obtained by solving a chemical Master equation, see *Materials and Methods* and SI for details. $J_{\max}$ is taken from SI Appendix, Table S2 ("ox-sc$^a$").

ages of the heme groups. Hence, the $\beta$ values reported here are about a factor of two larger than the typical range for through-protein tunneling, 1.0-1.5 Å$^{-1}$(25), whilst the tunneling distance for each consecutive hop is about a factor of 2 or more smaller than for typical through-protein tunneling processes. As is well known, over distances of several nanometers and beyond multi-step hopping outcompetes 1-step tunneling due to its favourable $1/R$ scaling compared with exponential scaling for 1-step tunneling.(25) There are amino acid side chains that bridge the gap between co-planar and T-shaped heme pairs (e.g. between hemes 1 and 6, ILE252, LEU571 in MtrC and PRO540, PRO243 in MtrF), implying that amino acid-mediated heme-to-heme tunneling could be an alternative mechanism. However, using pathway calculations(33, 34), we found that the dominant through-space tunneling path always gave couplings at least an order of magnitude higher than any amino acid-mediated pathway, which rules out this alternative mechanism, at least at the level of pathway calculations.

**Reorganization free energy and driving force.** We have calculated the reorganization free energy $\lambda$ for ET between all adjacent hemes in MtrC using MD simulations. For the purpose of deriving fit parameters for $\lambda$ in MHCs, we also computed $\lambda$ for the penta-heme MHC NrfB and take values for MtrF(35) and STC(32) from our previous work. We find that the values for all four proteins fall in the range 0.7-1.1 eV with values for MtrC being slightly smaller on average than for MtrF (see Table S1). Interestingly, the dominating outer-sphere reorganization free energy due to protein and solvent, $\lambda_o$, does not correlate with the solvent accessible surface area (SA) of the heme pairs (fig. 3A), but can be well described by Marcus' continuum formula if the static dielectric constant is assumed to be a linear function of the SA, $\epsilon_s(SA) = a + bSA$, $a, b$ constants. The smallest mean deviation with respect to $\lambda_o$ from MD is obtained for $a = 5.18$, $b = 0.016$ Å$^{-2}$ and an effective heme radius $r = 4.6$ Å(using an optical dielectric constant, $\epsilon_{op} = 1.84$(37)), see fig. 3B. This gives $\epsilon_s$ values between 6 (for the buried heme pair 1-3 of MtrC) and 14 (for the strongly solvent exposed heme pair 10-9 of MtrF).

ET driving forces are calculated for the all-ox redox state of MtrC using molecular dynamics combined with thermodynamic integration. The resultant free energy profile for ET along the heme chains (Table S1) is qualitatively similar to the one reported recently by Barrozo *et al.* for the same redox state, denoted "Electron hopping regime" in their work(27), and is not further discussed here. For MtrF, ET driving forces are taken from our previous work(26).

**Electron flux through MtrC and MtrF.** The computed electronic couplings, reorganization free energies and driving forces are used to calculate the non-adiabatic (Marcus) rate constants for all heme-to-heme ET steps. They are used as an input for a chemical Master equation for electron hopping, which we solve to obtain the maximum, protein-limited electron flux through MtrC and MtrF. Briefly, we assume fast and irreversible electron input in a given terminal heme site, e.g. heme 10, and electron output from another terminal heme site, e.g. heme 5. The electron population of each single heme, which can take values between 0 (fully oxidized) and 1 (fully reduced), is determined subject to the condition of steady-state electron flux through the protein. Similar flux calculations are carried out for the reverse direction along the octa-heme chain and for ET from heme 10 and heme 5 to the side exits heme 7 and 2, respectively. Further details on the calculations can be found in the SI Appendix.

The results are illustrated in fig. 4 for MtrC (panel A) and MtrF (panel B). The heme-to-heme rate constants are proportional to the width of the arrows connecting hemes and the protein-limited electron flux for all 12 possible flow directions across MtrC and MtrF are shown in the insets (in powers of 10 s$^{-1}$). The rate constants along the octa-heme chains of MtrC and MtrF span four orders of magnitude, from $\approx 10^5 - 10^9$ s$^{-1}$, and the electron flux is $\approx 10^5$ s$^{-1}$ in both the $10 \to 5$ and $10 \leftarrow 5$ directions. Electron flow from hemes 10 or 5 to the side exits 7 and 2 is similarly fast as along the octa-heme main chain, about $10^5$ s$^{-1}$, except for $10 \to 7$ due to the relatively high reduction potential of heme 7. But the latter is subject to uncertainty as discussed previously(26, 27) and may be overestimated. The reverse flow from the side exits 7 or 2 to 10 and 5 is somewhat slower, typically about $10^4$ s$^{-1}$, due to successive up-hill steps involving co-planar and T-shaped motifs. Similar results are obtained when the sets of reduction potentials from Barrozo *et al.* are used(27), with deviations of typically less than an order of magnitude (see

SI Appendix, Table S2). Overall, our results indicate that MtrC and MtrF conduct electrons about equally well along their main axis and in perpendicular directions with little or no directional bias.

Whilst the electron flux (with all heme side chains included) is remarkably similar for all directions, the electron flux enhancement due to the side chains is not the same in every direction - on the contrary, depending on the number of co-planar and T-shaped heme pairs and their free energies for a given flow direction, the flux enhancement varies from between a factor of 2 ($10 \rightarrow 7$ in MtrF, 1 T-shaped pair) to a factor of $\approx 10^3$ ($10 \rightarrow 5$ in MtrC, 1 co-planar and 2 T-shaped pairs). Without the Cys-mediated coupling enhancement, the co-planar or T-shaped heme pairs in the middle of the protein limit the electron flux, whereas with coupling enhancement these ET steps become similarly fast as ET between stacked heme pairs. In this case there is no longer a clearly flux-limiting ET step; the two slowest steps are within an order of magnitude.

**Discussion.** Although direct experimental estimates for heme-to-heme ET rate constants in solvated single-molecule MtrC and MtrF have not (yet) been reported, measurements have been carried out on related systems that lend support to some of our results. First, Butt and co-workers investigated the MtrC containing MtrCAB protein complex inserted in a proteoliposome and adsorbed on a Fe(III)-oxide nano-particle(38). The electron flux from an excess soluble electron donor across the entire MtrCAB complex on to the oxide was determined to be $10^4\,\mathrm{s}^{-1}$. It was shown that the rate was limited by the heterogeneous ET step from the protein to the oxide, hence should be considered a lower bound to the protein-limited rate. Our estimate for the latter, $10^5\,\mathrm{s}^{-1}$, is thus in line with this experimental result.

Second, El-Naggar and co-workers recently reported electrochemical gating experiments on *Shewanella oneidensis* MR1 cells(39), which require the Mtr pathway cytochromes (in particular MtrC) for electron transfer to the electrodes. Measuring the conduction current as a function of temperature, Arrhenius behaviour was observed and the thermal activation energy for electron transport determined to be 0.29 eV. This compares very favourably with the calculated largest activation free energy for heme-to-heme hopping steps along the octa-heme chain, $\Delta A^{\ddagger} = (\lambda + \Delta A)^2/(4\lambda) = 0.33\,\mathrm{eV}$ in the $10 \rightarrow 5$ direction (heme pair 6-1), and $\Delta A^{\ddagger} = 0.29\,\mathrm{eV}$ in the $10 \leftarrow 5$ direction (heme pair 8-6).

A third type of experiment one could compare our results to are the $I-V$ measurements by Rosso, El-Naggar and co-workers on single MtrC(9) and MtrF(11) proteins using STM. Assuming the same hopping mechanism as for ET in solution(11, 28, 40, 41), we obtain currents of a few 0.1 nA at 0.5 V bias voltage for MtrC and MtrF, respectively, in good agreement with experiments(9, 11) (see SI Appendix, fig. S2). By contrast, without the Cys-mediated electronic coupling enhancement, the currents are two orders of magnitude too low. The favourable comparison with the STM currents should be considered with some caution, however, since a number of assumptions went into the modelling (see SI Appendix for discussion.) In this regard, we note that recent $I-V$ measurements on MtrF monolayer junctions reported temperature-independent transport, which is incompatible with thermally activated hopping(12). However, the experimental conditions in this latter study are quite different with respect to the above mentioned STM measurements (high-vacuum vs air, protein monolayer vs single molecule, suspended nanowire vs tip) which may tip the balance between different mechanisms.

Finally, we wish to investigate whether the popular pathway model(33, 34) can capture the rate enhancements due to the heme side chains as predicted by present DFT/POD calculations. To this end we have calculated the enhancement factor $r_{\mathrm{pw}} = \langle |H_{\mathrm{ab}}^{\mathrm{pw}}|^2 \rangle / \langle |H_{\mathrm{ab}}^{\mathrm{ts}}|^2 \rangle$, where $H_{\mathrm{ab}}^{\mathrm{pw}}$ and $H_{\mathrm{ab}}^{\mathrm{ts}}$ are the pathway (pw) coupling matrix elements for heme-to-heme electron tunneling along the strongest coupling path in the large QM model (typically through-space via side chains) and in the minimum QM model (through-space edge to edge), and $\langle \cdots \rangle$ denotes the thermal average over MD snapshots. In the following we compare $r_{\mathrm{pw}}$ with $r_{\mathrm{dft}}$ defined in section *Results*. We find values $r_{\mathrm{pw}} = 2200$ ($r_{\mathrm{dft}} = 2500$) for the co-planar heme pair 1-6 and 120 (38) and 170 (11) for the T-shaped heme pairs 8-6 and 1-3 for pathway (DFT/POD) calculations on MtrC, and similar results for MtrF (see SI Appendix, Table S3). The agreement is excellent for the heme pair 1-6 that limits the overall electron flow and semi-quantitative overall. Yet, the pathway model incorrectly predicts rate enhancements of the same size if the S atom of the Cys linkages is changed into $CH_2$(32). This problem could be addressed by including chemical specificity in a refined version of the pathway model.

**Concluding Remarks.** We found that both MtrC and MtrF form a tri-furcated electron conduit that channels electrons with similar efficiency in perpendicular ($10 \leftrightarrow 5$) and parallel ($7 \leftrightarrow 2$) directions relative to the outer-membrane. The electron flow is reversible implying that both proteins not only support electron export but also electron import, a feature that enables electrode-driven electrosynthesis of chemicals inside the bacterial cell(5–8, 42). The tri-furcation of the electron flow in MtrC and MtrF is achieved by two junctions in the middle of the protein comprised of heme pairs with relatively large edge-to-edge tunneling distances (T-shaped, co-planar). Intriguingly, our calculations indicate that the junctions do not slow down the electron flux because Cys linkages inserting in the space between these heme pairs significantly enhance electronic coupling by reducing the effective tunneling distance. The same effect has been observed before for the two T-shaped pairs in the smaller tetra-heme cytochrome STC(32) implying that the coupling enhancement could be an evolutionary design principle of significance to the entire class of multi-heme cytochromes.

We note that there is little difference in the protein-limited electron flow through MtrC and MtrF. While reorganization free energies are slightly lower in MtrC than in MtrF, in line with reduced solvent accessible surface area of its hemes, no significant differences in electronic coupling are discernable. Thus, from the perspective of redox function our characterization suggests that MtrC can be replaced by MtrF, as in fact observed experimentally.(29, 30) The higher expression levels of MtrCAB relative to MtrFDE at low $O_2$ concentrations(43) is thus more likely related to a genetic origin rather than electron transfer function.

Efficient ET in MtrC in the direction parallel to the outer-membrane is one of the prerequisites for micrometer-long electronic conduction along cellular appendages as observed in Ref.(22) Whether the intraprotein ET in MtrC studied here or

the interprotein ET between adjacent MtrCAB complexes is the flux-limiting process remains to be investigated. According to the cryomicroscopy study of Ref. (19) adjacent MtrCAB complexes may be separated by more than 30 nm, as depicted in fig. 1E. Hence, the answer to this question will depend on the diffusivity of the MtrCAB protein complex in the outer membrane and the kinetics of the interprotein ET step between two interacting MtrC proteins. Modelling of these processes will require a suitable respresentation of MtrCAB, possibly coarse-grained to study its diffusivity on long time scales, as well as an atomistic structure of the MtrC-MtrC interface.

## Materials and Methods

Molecular dynamics simulations were carried for aqueous MtrC and NrfB at room temperature starting from the crystal structures pdb id 1M1Q(44) and pdb id 2OZY(13), respectively, using the AMBER03 force field(45) and the TIP3P water model(46). Driving forces ($\Delta A_{ji}$) for MtrC and reorganization free energies ($\lambda$) for MtrC and NrfB were obtained from MD stimulation as described for MtrF(26, 35). Heme-heme electronic coupling matrix elements ($H_{\mathrm{ab}}$) were calculated for MtrC and MtrF as described in Ref. (32) using the projection operator-based diabatization (POD) method(47, 48) in combination with a modified PBE functional where 50% GGA exchange is replaced by Hartree-Fock exchange. This method showed excellent performance(48) against high-level ab initio reference values on dimers of the HAB11 database.(49). Full computational details can be found in the SI Appendix.

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