Electronic Supplementary Material (ESI) for Dalton Transactions.

Diving into the redox properties of *Geobacter sulfurreducens* cytochromes: a model for extracellular electron transfer

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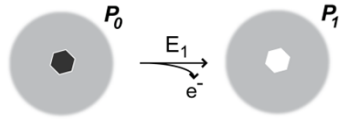
Electronic Supplementary Information

Fig. S1 Electronic distribution scheme for monoheme (A), diheme (B) and triheme (C) cytochromes showing the possible microstates in each situation. The inner hexagons circles represent heme groups, which can be either reduced (black hexagons) or oxidized (white hexagons). The microstates are grouped according to the number of oxidized hemes in each oxidation stage. The equations that allow to determine the reduced fractions are also indicated. For multiheme cytochromes containing higher number of hemes the distribution can be easily scaled-up. The total number of microstates and oxidation stages would be given by 2^N and N+1, respectively (N representing the total number of heme groups).

A



Fully reduced (S_0) Fully oxidized (S_1)

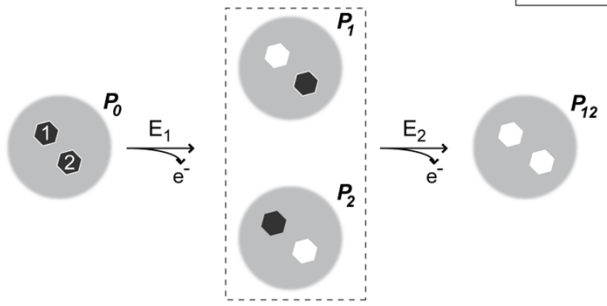
$$[S_1] = [S_0]\ exp\left(\frac{(E-E_1)\,F}{RT}\right)$$

$$[S_2] = [S_1]\ exp\left(\frac{(E-E_2)\,F}{RT}\right)$$

$$[S_3] = [S_2]\ exp\left(\frac{(E-E_3)\,F}{RT}\right)$$

$$\textit{Reduced fraction} = \frac{[S_0]}{[S_0] + [S_1]}$$

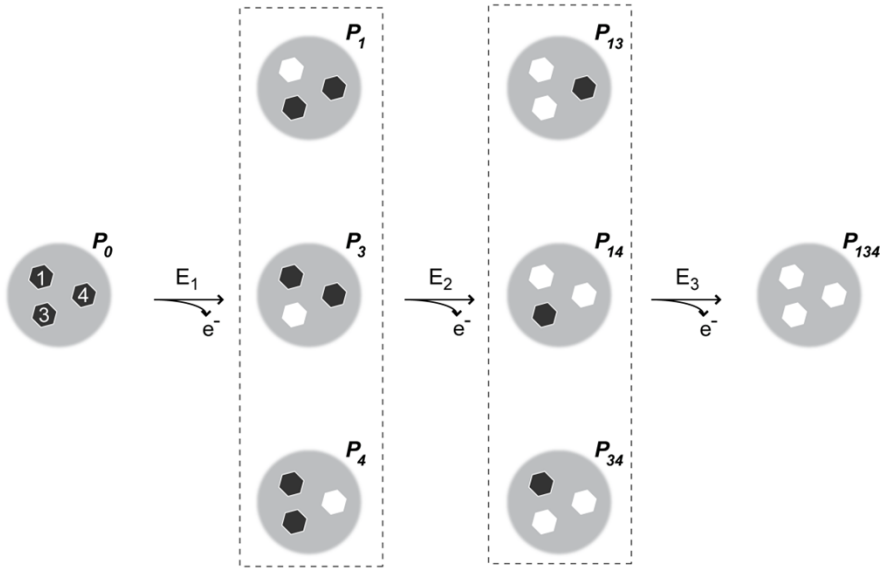
B



Fully reduced (S_0) Oxidation stage 1 (S_1) Fully oxidized (S_2)

$$\textit{Reduced fraction} = \frac{2[S_0] + [S_1]}{2([S_0] + [S_1] + [S_2])}$$

C



Fully reduced (S_0) Oxidation stage 1 (S_1) Oxidation stage 2 (S_2) Fully oxidized (S_3)

$$\textit{Reduced fraction} = \frac{3[S_0] + 2[S_1] + [S_2]}{3([S_0] + [S_1] + [S_2] + [S_3])}$$

Materials and Methods

Expression and purification of PpcC and GSU1996

The cytochrome PpcC and GSU1996 were heterologously expressed in *Escherichia coli* BL21 (DE3) cells holding the plasmid pEC86 which contains the *ccm* genes essential for the maturation of *c*-type cytochromes in *E coli*, as previously described[1,2]. *E. coli* BL21 (DE3) cells were then co-transformed with the plasmid pCK32 containing the gene code for each cytochrome. Bacteria were grown at 30 ºC in 2x yeast extract–tryptone medium containing 34 μg/mL chloramphenicol and 100 μg/mL ampicillin, since the plasmids pEC86 and pCK32 are resistant to these antibiotics, respectively. Protein expression was induced with 10 μM (PpcC) and 20 μM (GSU1996) isopropyl β-D-thiogalactoside (IPTG). The cytochromes were purified in two steps: (i) cation exchange chromatography, using to two 5 mL Econo-Pac HighS cartridges (BioRad) connected together equilibrated with 10 mM Tris−HCl (pH 8.5) and eluted with a NaCl gradient from 0 to 300 mM in 10 mM Tris−HCl (pH 8.5) and (ii) gel filtration chromatography using a Superdex 75 (GE Healthcare) equilibrated with 100 mM phosphate buffer pH 8.0. The purity of the samples was confirmed by SDS-PAGE stained with Coomassie blue (Sigma).

Redox titrations followed by visible spectroscopy.

The redox titrations of purified cytochromes PpcC and GSU1996 were carried out inside an anaerobic chamber (MBraun) kept at < 1 ppm oxygen at 298 K, as previously described[3]. Proteins samples with 18 μM protein concentration were prepared at pH 7.0 in 32 mM sodium phosphate buffer with NaCl (100 mM final ionic strength). To check

for hysteresis, each redox titration was performed in both oxidative and reductive directions, using sodium dithionite and potassium ferricyanide solutions as reductant and oxidant, respectively. To ensure a good equilibrium between the redox centers and the working electrode, a mixture of the following redox mediators was added to the solution, all at approximately 1.5 μM final concentration: gallocyanine, methylene blue, indigo tetrasulfonate, indigo trisulfonate, indigo disulfonate, 2-hydroxy-1,4-naphthoquinone, anthraquinone-2,6-disulfonate, anthraquinone-2-sulfonate, safranine O, neutral red, benzyl viologen, diquat and methyl viologen. For cytochrome GSU1996 the following additional mediators were also used: p-benzoquinone, 1,2-naphtoquinone-4-sulfonic acid, 1,2-naphtoquinone, trimethylhydroquinone, phenazine methosulphate, and phenazine ethosulphate. The mediators were chosen according to the redox ranges of the proteins in accordance with the procedures defined in the literature[4] and covered the potential range of -440 to +80 mV. The reduced fraction of the proteins was determined by integrating the area of the α-peak (552 nm) above the line connecting the flanking isosbestic points (543 and 559 nm) to subtract the optical contribution of the redox mediators, as previously described[3]. The experiments were performed at least two times, and the reduction potentials (relative to standard hydrogen electrode, SHE) were found to be reproducible within ± 5 mV.

References

1. P. R. Pokkuluri, Y. Y. Londer, N. E. Duke, M. Pessanha, X. Yang, V. Orshonsky, L. Orshonsky, J. Erickson, Y. Zagyansky, C. A. Salgueiro and M. Schiffer, *J. Struct. Biol.*, 2011, **174**, 223.
2. P. R. Pokkuluri, Y. Y. Londer, X. Yang, N. E. Duke, J. Erickson, V. Orshonsky, G. Johnson and M. Schiffer, *Biochim. Biophys. Acta*, 2010, **1797**, 222.
3. L. Morgado, M. Bruix, V. Orshonsky, Y. Y. Londer, N. E. Duke, X. Yang, P. R. Pokkuluri, M. Schiffer and C. A. Salgueiro, *Biochim. Biophys. Acta*, 2008, **1777**, 1157.
4. P. L. Dutton, *Methods Enzymol.*, 1978, **54**, 411.