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Commentary

Making protons tag along with electrons

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Every living cell needs to get rid of leftover electrons when metabolism extracts energy through the oxidation of nutrients. Common soil microbes such as *Geobacter sulfurreducens* live in harsh environments that do not afford the luxury of soluble, ingestible electron acceptors like oxygen. Instead of resorting to fermentation, which requires the export of reduced compounds (e.g. ethanol or lactate derived from pyruvate) from the cell, these organisms have evolved a means to anaerobically respire by using nanowires to export electrons to extracellular acceptors in a process called extracellular electron transfer (EET) [1]. Since 2005, these nanowires were thought to be pili filaments [2]. But recent studies have revealed that nanowires are composed of multiheme cytochromes OmcS [3,4] and OmcZ [5] whereas pili remain inside the cell during EET and are required for the secretion of nanowires [6]. However, how electrons are passed to these nanowires remains a mystery (Figure 1A). Periplasmic cytochromes (Ppc) called PpcA-E could be doing the job, but only two of them (PpcA and PpcD) can couple electron/proton transfer — a necessary condition for energy generation. In a recent study, Salgueiro and co-workers selectively replaced an aromatic with an aliphatic residue to couple electron/proton transfer in PpcB and PpcE (*Biochem. J.* **2021**, *478* (14): 2871–2887). This significant *in vitro* success of their protein engineering strategy may enable the optimization of bioenergetic machinery for bioenergy, biofuels, and bioelectronics applications.

Although most EET studies remain focused on electrons, protons play a very important role, not only in bacterial energy generation, but also in the electronic conductivity of proteins. Proteins are typically considered electronic non-conductors, but recent mechanistic studies on model systems with atomic-resolution protein structures are helping to identify design principles to engineer electronic conductivity in proteins for a wide range of applications, including artificial photosynthesis, biocatalysis, prevention of oxidative damage, and nucleic acid biosynthesis [7]. For example, through measurements of the intrinsic electron transfer rate, Shipps et al. [7] recently showed that both the energetics of a glutamine (proton acceptor) and its proximity to a neighboring tyrosine (proton donor), regulate the hole transport over micrometers in amyloids through a proton rocking mechanism. Therefore, it is very important to couple electron/proton transfer to accelerate EET and for the development of electronically conductive protein-based biomaterials.

Multiheme cytochromes represent ideal biomaterials to achieve this goal because of their wide redox potential window. In addition to the abovementioned natural nanowires made up of polymerized cytochromes, some cytochromes can form conductive structures when crystallized but their electron transfer rate remains low [8]. Salgueiro and co-workers have demonstrated that systematically mutating key residues to alter the heme environment in such cytochromes can tune their redox potential and dial up or down the coupling of electron and proton transfers. Such innovative approaches can accelerate EET by improving conductivity of natural or synthetic protein nanowires.

During cellular respiration, protons from oxidative metabolism are thought to accumulate and acidify the cytoplasm under EET-respiring conditions, thereby dissipating the electrochemical gradient across the inner membrane needed for ATP synthesis [9]. This proton accumulation causes significantly slower bacterial growth compared with when a soluble electron acceptor (e.g. fumarate) is

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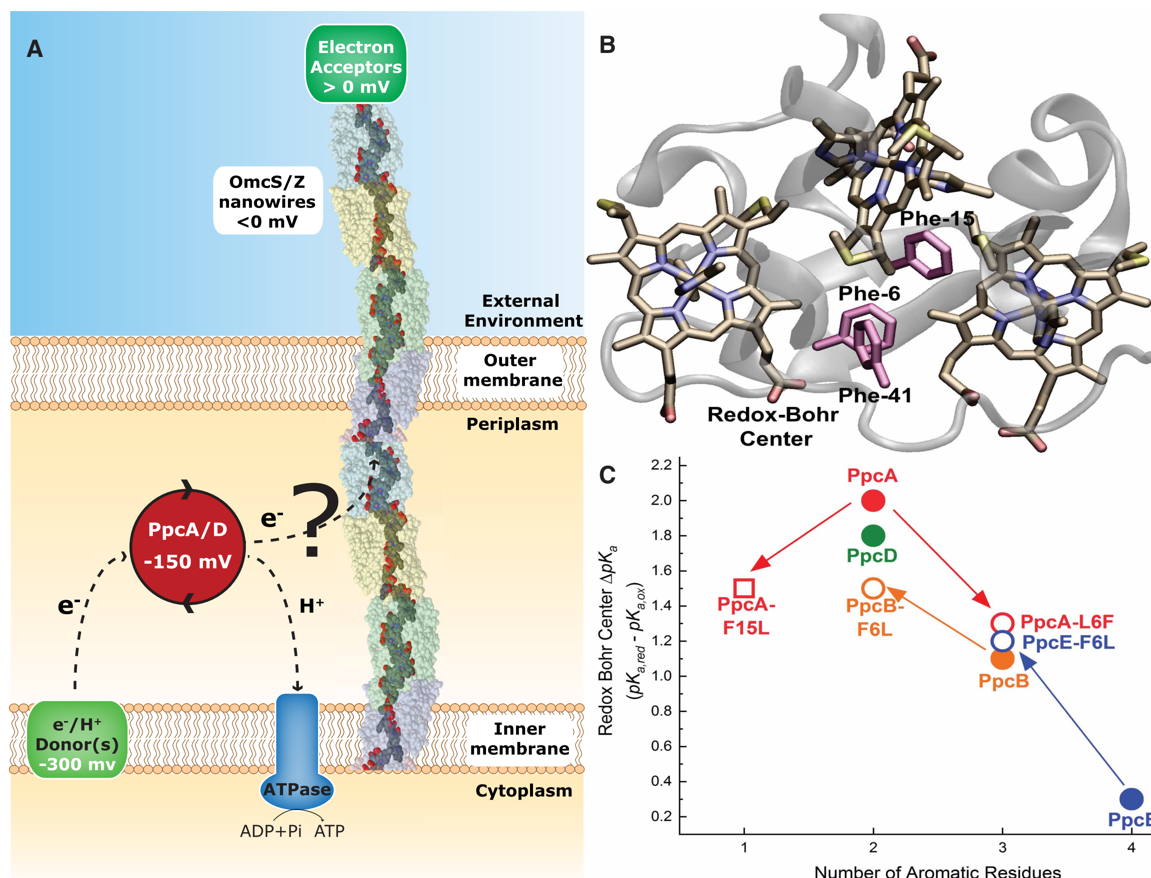

Figure 1. Proton coupled electron transfer is important for bacterial extracellular electron transfer via multiheme cytochromes.

(**A**) Hypothetical model for EET in *G. sulfurreducens*, highlighting the important role of PpcA (and homologs) in ferrying electrons across the periplasm to the cytochrome nanowires. (**B**) Structure of PpcB (PDB 3BXU) [13] as a representative of the PpcA family. The Phe residues at positions 6 and 15 were the subject of mutagenesis experiments in PpcA [21,22], but no experiments have so far been reported on the role of Phe-41 (or the additional Tyr-45 in PpcE). PpcB was chosen to represent the family to emphasize the location of the aromatics at position 6 and 15 found in this isoform. (**C**) Correlation of the ΔpK_a of the redox-Bohr center to the number of aromatic residues in the PpcA family member. The filled circles, or open circles and squares correspond respectively to the naturally occurring proteins and engineered single-point mutants. No single-point mutant has so far been analyzed for PpcD. Red, orange, green, and blue are used for PpcA, PpcB, PpcD and PpcE, or their mutants, respectively. The figure is based on data presented in Table 3 of Ref. [21], and Ref. [22].

available, even when reduction in an extracellular acceptor (e.g. Fe(III) citrate) can provide more energy [9]. In fact, a metabolic modeling study [9] concluded that bacterial growth would be impossible with EET unless unknown mechanisms contributed to the consumption of cytoplasmic protons. If these mechanisms were identified, they may be rationally engineered to support greater bacterial growth rates for applications where this is an important parameter.

A possible mechanism to discharge cytoplasmic protons under EET-respiring conditions emerged from Salgueiro and co-workers' efforts to systematically characterize the structural [10–14] and thermodynamical [13,15,16] properties of almost every member of a protein family known to be critical for EET in *G. sulfurreducens*. Metabolic electrons must cross the periplasm as part of the exodus from the cell in EET (Figure 1A). Five constitutively expressed proteins of the PeriPlasmic Cytochrome A (PpcA) family ferry these electrons from multiheme complexes in the inner-membrane to ones in the outer-membrane. The PpcA family members, designated Ppc- A through E, are each 10 kDa triheme cytochromes that have one of the smallest (∼24 : 1) amino acid-to-heme ratios [17]. A structure of PpcB is shown in Figure 1B as a representative of the family.

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From the perspective in Figure 1B, the hemes are positioned at the apices of an inverted V-shape structure. All PpcA homologs have a conserved positive electrostatic potential near the left-most heme in Figure 1B, and the lowest structural similarity near the right-most heme [14]. This observation led to the hypothesis that functional heterogeneity within the PpcA family allows these proteins to discharge electrons from a common electron donor to a diversity of electron acceptors [14]. Consistent with this idea, a mutagenesis study found that PpcE is only expressed under Fe(III) citrate growth conditions, whereas PpcA, PpcB, and PpcD are expressed when either Fe(III) citrate or oxide are available [18]. It is currently not known if PpcA homologs are physiological electron donors/acceptors for one another. Given similar or partly overlapping redox-active ranges, PpcA homologs may work together in a relay-fashion to get electrons across the periplasm. Protein–protein interaction studies are needed to test this hypothesis.

Salgueiro and co-workers found that some family members had the unique ability to couple proton translocation to electron transfer. In particular, the A and D isoforms could uptake a strongly reducing electron ($\sim$−167 mV) and weakly acidic proton (pH $\sim$ 8), and transmit to a downstream protein partner a de-energized electron ($\sim$−100 mV) and a sufficiently acidic proton (pH $\sim$ 7) to be deposited in the periplasm [16]. This energy transduction mechanism for coupling electron and proton (e^-/H^+) transfer made Ppc- A and D strong candidates for contributors to the creation of membrane potential [19].

Given the high level of sequence identity (45–77%) [14] and structural conservation within the PpcA family, the observation of different functional activities among the homologs has posed a series of fundamental questions, including: What is the origin of e^-/H^+ energy transduction in Ppc- A and D? Why is the ability reduced in Ppc- B and E? And can the e^-/H^+ transfer functionality be enhanced in Ppc- B and E so these isoforms can also contribute to the membrane potential? Aspects of all these matters have now been illuminated by Salgueiro and co-workers.

Close inspection of the PpcA homologs revealed that the B and E isoforms, which are less efficient at coupling e^-/H^+ transfer, are both mutated at the fifth, sixth, and 28th residues (numbers correspond to the PpcA protein) relative to the A and D isoforms that perform energy transduction. Of these mutations, isoforms B and E only share the same residue (Phe) at the sixth position. The fifth and 28th residues are Thr and Ala or Leu and Arg in PpcB and PpcE, respectively. Ppc- A and D, by contrast, feature a Leu at position 6. These observations suggested that an aliphatic-to-aromatic mutation regulates the degree of coupling between e^-/H^+ transfer in the PpcA family. Two further observations by Salgueiro and co-workers seemed to support this surprising hypothesis: (1) Residue 6 in all isoforms is spatially near a heme propionate whose pK_a is coupled to redox transitions in the protein (i.e. it is a redox-Bohr center), and (2) the degree to which e^-/H^+ transfers are coupled — measured by the change in the pK_a of the redox-Bohr center between the fully reduced and fully oxidized states of the protein — is inversely related to the number of aromatic residues in a PpcA homolog (Figure 1C). Ppc- E, B, D, and A respectively have 4, 3, 2, and 2 aromatic groups, and ΔpK_as of 0.3, 1.1, 1.8, and 2.0. (PpcC, which has a Tyr at the sixth position, could not be thermodynamically characterized by nuclear magnetic resonance techniques because of conformational heterogeneity during the redox cycle of the protein).

To test how the identity of residue 6 regulates the coupling of e^- and H^+ transfers, Salgueiro and co-workers mutated Leu-6 in PpcA to Phe and Phe-6 in PpcB and E to Leu [20]. These mutations fortunately did not appreciably change the global fold or heme core architecture of the proteins, the working functional range for heme redox potentials, or the order in which the heme groups are oxidized at physiological pH. Instead, the mutations substantially changed, as hypothesized, the coupling of redox and acid-base equilibria, as shown in Figure 1C with the mutation-induced shifts (filled to empty symbols) in ΔpK_a values. The L6F mutation in PpcA lowered ΔpK_a by 0.6 units (Figure 1C; filled to empty red circles), whereas the F6L mutation in PpcB/E increased ΔpK_a by 0.4–0.9 units (Figure 1C; filled to empty orange and blue circles respectively). Figure 1C also indicates the ΔpK_a value for wild-type PpcD with a green filled circle. Thus, the strategic reversal of an evolutionary Leu-to-Phe mutation in Ppc- B and E restored coupled e^-/H^+ activity in these proteins. Whether or not expression of these engineered proteins *in vivo* will contribute to the generation of membrane potential and thereby biomass growth awaits experimental verification. If an enhancement in membrane potential is confirmed, however, the work by Salgueiro and co-workers will mark a milestone in our ability to understand and utilize microbial EET.

An important physical insight is that native and single-point mutants of PpcA homologs having the fewest aromatic residues also have the largest ΔpK_a values for the redox Bohr center (Figure 1C). The precise physical basis for this correlation, and its breakdown for a Phe-to-Leu mutation at position 15, instead of position 6, in PpcA (red filed circle to empty red square), remain to be elucidated.

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From a systematic structural and thermodynamic characterization of a protein family to the mutagenesis-driven exploration of hypothesized roles for functional differences among the homologs, Salgueiro and co-workers' rational protein engineering strategy has advanced our molecular understanding of EET mechanisms. Beyond the immediate importance of insights regarding the regulatory role of a non-polar residue in the coupled e^{-}/H^{+} transfer activity of PpcA homologs, their approach can be more generally applied to disentangle and design electrical network components in microbial catalysts. We look forward to how the authors will apply this strategy to answer the interesting bio- and physical-chemical aspects emerging from their work.

On the biochemical side, why does *Geobacter* not already exploit the F6L point mutation in all PpcA family members if doing so would increase the proton motive force for ATP synthesis and thereby increase biomass production? Salgueiro and co-workers seem to assume that the lessened e^{-}/H^{+} transferring ability of PpcB/E relative to PpcA/D constitutes a metabolic bottleneck for energy generation, but this assumption is a yet untested hypothesis. Alternatively, the PpcA homologs have been optimally tuned through evolution to function in a complex network of interactions that interface invariant metabolic reactions in the cytoplasm with a diversity of extracellular environments encountered by *Geobacter*. In this network perspective, optimization of the functionality of PpcA homologs in isolation may be ineffective, or possibly even detrimental for the overall system: Homogenizing PpcA family members so they all perform e^{-}/H^{+} transfers may decrease the robustness of the EET system to cope with variations within or between different habitats. It is also possible that there may be no evolutionary pressure at present for *Geobacter* to expend resources for the further optimization of its EET pathways. Importantly, the last two of these possible explanations for the absence of the pro-$e^{-}/H^{\pm}$-coupling mutation in PpcB/E imply that new strategies are required to rationally optimize *Geobacter* for its natural habitats over what evolution has already achieved.

Salgueiro and co-workers stress that the engineered e^{-}/H^{+}-coupling PpcB/E-F6L mutants are particularly promising for expression in genetically stripped-down strains of *Geobacter* that are more suitable for incorporation into hybrid bio-inorganic devices. stripped-down strains do not have the complexity of redundant EET pathways that can obscure the influence of the protein engineering. If *in vivo* expression of PpcB/E-F6L is achieved in a stripped-down *Geobacter* strain, the magnitude of the change in inner-membrane potential or biomass growth will be very instructive for assessing the practicality of improving organismal-level EET by modifying individual parts of an EET pathway. If increased biomass production is realized with expression of the PpcB/E-F6L *versus* the wild-type proteins, the rational bottom-up optimization of *Geobacter* for technological applications — starting from a genetically stripped-down background — would be shown to be a viable strategy. It is of interest in this context to test if stripped-down *Geobacter* strains only expressing PpcB or PpcE from the PpcA family would evolve the F6L mutation under laboratory or environmental conditions without rational intervention.

Even if the protein engineering strategy is successful for technological applications of *Geobacter*, however, much will otherwise remain unclear about the physiological role for differing degrees of e^{-}/H^{+} coupling among PpcA family members in the wild-type organism. Are the PpcA family members undergoing convergent or divergent evolution with respect to the ability to couple e^{-}/H^{+} transfers? Either way, what are the evolutionary pressures that make the expression of five proteins with partially overlapping redox and proton equilibria advantageous? These are only a few of the outstanding questions on the topic.

On the physical chemistry side, the lesson from studies of e^{-}/H^{+} activity in the PpcA family is that non-polar residues should not be viewed as just space-filling packing material; they can have a profound regulatory role, albeit indirectly, on the functional mechanism of a protein. It would be interesting for future work to explore how a Tyr at the sixth position, as in the yet-thermodynamically uncharacterized PpcC, impacts e^{-}/H^{+} coupling in contrast with the Phe of Ppc- B and E. Does the presence of an aromatic group still suppress coupled e^{-}/H^{+} activity, or does the polarity and H-bonding capability of the hydroxyl group introduce new effects on the redox-Bohr center altogether? Also, what exactly is the physical basis for the inverse correlation (Figure 1C) between the ΔpK_a of the redox-Bohr center and the number of aromatic groups? Why does the correlation not hold for the PpcA–F15L mutant? Is this correlation more generally applicable to other proteins as a design rule for rationally introducing or modulating proton-coupled electron transfer activity? We thus eagerly await future studies that address these questions.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviation

EET, extracellular electron transfer.

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