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Review

Multi-heme proteins: Nature's electronic multi-purpose tool☆

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ABSTRACT

While iron is often a limiting nutrient to Biology, when the element is found in the form of heme cofactors (iron protoporphyrin IX), living systems have excelled at modifying and tailoring the chemistry of the metal. In the context of proteins and enzymes, heme cofactors are increasingly found in stoichiometries greater than one, where a single protein macromolecule contains more than one heme unit. When paired or coupled together, these protein associated heme groups perform a wide variety of tasks, such as redox communication, long range electron transfer and storage of reducing/oxidizing equivalents. Here, we review recent advances in the field of multi-heme proteins, focusing on emergent properties of these complex redox proteins, and strategies found in Nature where such proteins appear to be modular and essential components of larger biochemical pathways. This article is part of a Special Issue entitled: Metals in Bioenergetics and Biomimetics Systems.

1. Introduction: types of multi-heme proteins used in nature

While biology is efficient at using metals to achieve electron transfer and catalysis, one of the most prevalent metal cofactors is heme iron [1]. The breadth of heme protein structure and function is an immense subject, even when considering only the diversity of proteins and enzymes that contain a *single* heme cofactor [2]. Here, we will focus on the so-called multi-heme proteins that contain multiple units of heme, and in particular heme proteins that can be described as multi-heme cytochromes *c*, where the vinyl groups of iron-protoporphyrin IX are attached to the two cysteine side chains of a $-CX_nCH-$ motif by enzymatic machinery (for bacteria, this has been previously reviewed [3]), where the His residue serves as a proximal heme ligand. Typically, in such motifs, there are two variable residues between the cysteine positions [4], though other *c*-type heme attachment motifs are known, including $-CX_3CH-$ and $-CX_4CH-$ [5], as well as a $-CX_{15}CH-$ motif that requires a dedicated maturation enzyme system [6,7]. However, in the simplest cases, the presence of the $-CXXCH-$ motif itself can be used as sequence-based diagnostic for the *c*-type covalent attachment of heme units in the context of bacterial organisms. In all cases presented here, multiple heme units appear to be essential for their role in supporting electron transfer chemistry within or between proteins and enzymes. Electron transfer is essential for countless biological processes. Efficient electron transfer occurs when the free energy for the electron to be transferred from donor to acceptor is negative meaning that the reduction potential of an intermediary must be fine-tuned to be in between those of the donor and the acceptor. Heme iron reduction potentials can be affected by ligand type, coordination geometry, and solvent accessibility as well as the pH of the environment [8–11]. In electron transfer through proteins, the distance between cofactors plays a critical role in how quickly electrons are passed. To overcome the challenge of long-range electron transport or multi-electron reactions, Nature has evolved chains of redox cofactors such as iron sulfur (Fe–S) clusters or heme groups, thus enabling electrons to be passed across membranes. With respect to these issues, here we will observe that multi-heme proteins display even more elaborate tricks in their capacity for redox chemistry.

Previous reviews of multi-heme proteins or cytochromes have highlighted their evolutionary relationships and the potential for emerging chemistry [12,13], while here we will examine recent, emergent properties that appear to be found in multi-heme proteins and enzymes, considering in turn three different abilities where Nature is expert and Man is novice: redox communication and conformational changes of protein structure (bacterial cytochrome *c* peroxidases), long-range electron transfer through the deployment of many heme cofactors (the multi-heme cytochromes of dissimilatory metal reduction), and storage of multiple reducing and oxidizing equivalents (hydroxylamine oxidoreductase and cytochrome c_{554}). Sadly, many multi-heme proteins and enzymes (e.g. octaheme tetrathionate reductase [14],

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octaheme nitrite reductase [15,16], thiosulfate dehydrogenase [17]) are beyond the scope of our current review. Instead, here we will highlight the wealth of biochemical and biophysical information that is now available from increasingly divergent multi-heme proteins, while also underscoring the voids in our understanding of chemistry which need to be filled.

2. Redox communication

2.1. A conversation between cofactors: redox communication

Metalloproteins and metalloenzymes are efficient tools in biology using metals to achieve both electron transfer and catalysis. Electron transfer plays a central role in biological energy conversion, photosynthesis or respiration and also in the regulation of gene expression. Redox communication, in the form of oxidation and reduction reactions, is involved in electron transfer between the redox active cofactors within proteins and enzymes. The charge transfer is either initiated by low molecular weight redox mediators, including, but not limited to ubiquinol, oxygen/superoxide or the NAD^+/NADH couple, or by the direct interaction of redox partner proteins. Below we discuss examples of conformational changes associated with inter-protein electron transfer reactions from a partner (protein or mediator) into an enzyme and inter-cofactor electron transfer reaction.

2.2. Bacterial cytochrome c peroxidases

Bacterial diheme cytochrome *c* peroxidases (bCcPs) differ from the canonical monoheme peroxidases such as horseradish peroxidase (HRP) [18] and yeast cytochrome *c* peroxidase (yCcP) [19] in their heme cofactor content and catalytic peroxide reduction mechanisms. Found in the periplasmic space of many gram negative microorganisms, the bCcP family includes not only genuine bCcP enzymes—the main topic of this section—but also diheme orthologs such as MauG, a poor peroxidase that is required for the oxidative installation of the tryptophanyl tryptophane quinone (TTQ) cofactor found in methylamine dehydrogenase (recently reviewed in [20]). The two heme cofactors present in all members of the bCcP family are covalently bound within two separate cytochrome *c*-like domains. In all genuine bCcPs, the high-potential Met-His ligated heme, the H-heme (250–350 mV vs NHE) serves as the electron transfer site, accepting electrons from physiological or artificial electron donors. The other heme, the low-potential *bis*-His ligated L-heme (~−300 mV) serves as the site of peroxide reduction [21]. (In contrast, in the case of MauG the two hemes are known to be very close in potential, displaying redox anti-cooperativity [22].) In the as-isolated state both heme irons are in the ferric oxidation state. The high potential heme, which is not present in monoheme peroxidases, may be responsible for storing a second oxidizing equivalent during the catalytic reaction cycle and is hypothesized to mediate the transfer of electrons from electron donor proteins to the peroxidatic heme [23], potentially abrogating the need for radical-based intermediates in canonical bCcP enzymes.

2.2.1. Redox activation of CcP results in peroxide reduction

The majority of bCcPs are isolated in a catalytically inactive state where both hemes are in the ferric state, exemplified by the diheme peroxidase from *Pseudomonas aeruginosa* (*Pa*) [21,24]. For such bCcP enzymes, the absence of any reducing equivalents provided by an electron donor protein or small molecule redox mediators (such as ascorbate) results in both hemes remaining in the ferric oxidation state where the active site is in a *bis*-His ligated conformation, preventing binding of the substrate peroxide to the active site heme [24] (Fig. 1A, blue). The active form of the enzyme can be achieved by the introduction of one electron through the H-heme electron transfer site. This initiation of activation by a one electron reduction of the H-heme is termed "reductive activation": direct reduction of the H-heme causes local conformational changes that reorient the ligand groups on the low-potential heme, allowing for peroxide to access the active site [25] (Fig. 1A, gray). The resultant mechanistic implications for canonical bCcP enzymes are schematized in Fig. 1B, which suggests that by "banking" an electron prior to catalysis, the first kinetic intermediate after a peroxide substrate binds may be described formally as a ferryl peroxidatic iron, and a ferric high potential iron. There are notable exceptions to the apparent requirement for reductive activation, however, as the enzymes from *Nitrosomonas europaea* and *Methylococcus capsulatus* have both been reported to be constitutively active in the fully oxidized form [26,27]. Thus, the specific role for reductive activation is unclear, though it can be noted that the phenomena may be useful for microbial organisms that undergo fluctuations in the electron acceptors that are used, possibly to only use reducing equivalents from the cytochrome *c* pool, when the probability of forming hydrogen peroxide from missteps of respiration is high.

The precise means for redox communication between the heme units is not understood perfectly to date. Physiological electron donors to bCcPs are often small monoheme cytochromes *c*, or blue copper proteins such as azurin and pseudoazurin [28,29] that are capable of reducing the high-potential heme. The heme sites themselves are over 12 Å apart (edge-to-edge), such that electron transfer is still possible between the two heme cofactors via electron tunneling. Four possible through-bond routes for electron transport between the H- and L-hemes of the *Pa* CcP have been proposed [18,24] to date: (1) mediated by His201 adjacent to the H-heme via the protein backbone to the L-heme propionate [19]; (2) from the H-heme propionate, through a conserved calcium ion at the interface between the two heme domains, and then using the protein backbone to reach the L-heme [21]; (3) using ligating His261 to connect the H-heme propionate with Asn79 to then use the Ca^{2+} ion and the protein backbone to reach the L-heme [23]; and, (4) transfer from an H-heme propionate using Trp94 to an L-heme propionate (all residues mentioned use *Pa* numbering). However, only the last of these four hypotheses has been tested rigorously, where mutations made in Trp94 have resulted in inactive enzyme in steady-state analyses [30,31].

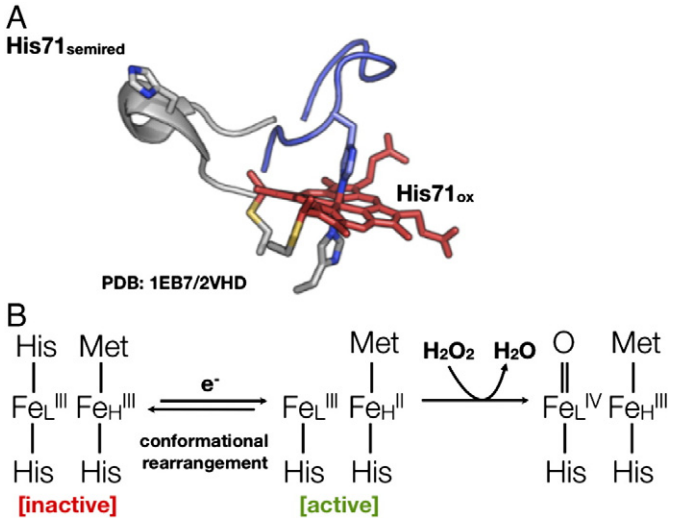

Fig. 1. A. The conformational switch found in the majority of known bCcP enzymes involves the reorganization of the distal face of the peroxidatic heme, as a function of the redox state of a high-potential heme, some 12 Å away. B. The mechanistic impact of reductive activation suggests that by "banking" an electron in the high potential center, the first kinetic intermediate needs to involve the build-up of a radical species.

A further complication in studies of bacterial CcPs stems from their native homodimeric nature, as all known bCcPs exist as homodimers in solution, regardless of whether or not the enzyme in question is of the canonical type requiring reductive activation [24,26,27,32–34]. The CcP from *Paracoccus denitrificans* shows a loss of activity upon dilution indicating that an equilibrium may exist between the monomer and the dimer, having the dimer as the active form [34], and dimerization has been further shown to be governed by the presence of a Ca^{2+} binding site within each protomer, though it is currently unclear whether Ca^{2+} induces dimerization or if Ca^{2+} binds after dimerization. It is known, however, that Ca^{2+} is essential for the reduction and hence activation of the enzyme, as shown with the CcP from *Paracoccus pantotrophus* [35]. In *Pseudomonas nautica*, Ca^{2+} is reported to be needed to induce conformational change around the L-heme [36]. In the *Pa*, *P. nautica*, and *Rhodobacter capsulatus* enzymes, the positive charge of the calcium ion is not balanced by any negatively charged amino acids, showing that it may be responsible for facilitating electron transport between the H- and L-hemes [24,30,32,36]. Recently, a charge reversal mutant of the enzyme from *Shewanella oneidensis* (*So*) has been reported that still maintains considerable activity, though it exists as a monomer in solution [37]. In the E258K mutant of the *So* enzyme, the same requirement for reductive activation is maintained, but the resulting $Fe_L^{III}Fe_H^{II}$ state required for catalysis (Fig. 1B) is much less kinetically stable than wild-type, emphasizing that bCcPs are sensitively tuned masters of redox communication and conformational control.

2.2.2. Redox related conformational changes in CcPs

X-ray crystal structures for a bCcP in the canonical *Pa*-like class of CcPs or the *Ne*-like class of CcPs have allowed for the comparison of structural changes due to different redox states for the enzymes. Crystal structures for both the oxidized and semireduced states of the CcPs from multiple organisms have allowed for an overall comparison between specific conformational changes in both the oxidized ($Fe_L^{III}Fe_H^{III}$), and semireduced ($Fe_L^{III}Fe_H^{II}$) states. The *Pa* enzyme is commonly used to demonstrate the comparison of the placement of three key loops in the oxidized state (shaded red, blue and green in Fig. 2A) against these same loops in the one electron reduced state [25] (Fig. 2B). Where Loop 1 (red) bears the universally conserved His residue that may serve as a ligand in the inactivated forms of bCcP, Loop 2 (blue) reorganization is required to form the distal pocket around the peroxidatic heme, while Loop 3 packs around the high-potential heme, and is presumed to present at least part of the surface responsible for interactions with electron donors. The structural changes shown in Fig. 2 appear to be uniform among the bCcP sub-family that require reductive activation: For example the oxidized *P. pantotrophus* (*Pp*) enzyme [38], a canonical family member, closely resembles the oxidized *P. aeruginosa* (*Pa*) enzyme [21] while the semi-reduced *P. pantotrophus* enzyme closely resembles the oxidized *N. europaea* enzyme [26] and the semi-reduced *Pa* enzyme. The oxidized *Pp* enzyme and the oxidized *Pa* enzyme are closely related in all conformations. There is also very close structural similarity between the mixed valent *Pp* enzyme and the oxidized *Ne* enzyme [39]. This structural similarity is consistent with the fully oxidized *Ne* enzyme being in an active state without the need for a prereduction step [26]. In both cases, there is open access to the active site in either the $Fe_L^{III}Fe_H^{II}$ form of the *Pa*-like enzymes, or the $Fe_L^{III}Fe_H^{III}$ state of the *Ne*-like enzymes. It is not known whether any redox state or other condition can compel the *Ne* enzyme to adopt the closed off, *bis*-His ligated state that is found in fully oxidized, canonical bCcP (as in Fig. 2A). Recent success in manipulating the apparent requirement for reductive activation has been reported recently through the studies of Einsle and co-workers, who have structurally and functionally characterized paralogs of the *Pa*-type of bCcP from *Geobacter sulfurreducens* (CcpA and a second diheme peroxidase, MacA) [40,41]. Indeed the CcpA protomer adopts the same fold as other canonical bCcP enzymes, displays the identical requirement for reductive activation, and has sequence elements in Loops 1, 2 and 3 that suggest a strong similarity to *Pa*-type of bCcP enzyme. While mutations in Loop 1 that would render the sequence more like the *Ne* enzyme (and thereby presumably convert the enzyme to be constitutively active) did not perturb the reactivity of CcpA, the S134V/P135K double mutant in Loop 2 did (in part) achieve *Ne* enzyme-like activity [40,42]. The resulting structure of the fully oxidized form of S134V/P135K shows a partially converted enzyme, where Loop 1 has shifted to an open conformation such that the substrate might bind, yet Loop 2 is only partially reorganized as it should be in semi-reduced bCcPs, and Loop 3 failed to convert to the required, semi-reduced conformation [40]. A further functional result of this partial inter-conversion between enzymatic conformers, was an apparent control of inter-cofactor electron transfer upon electrocatalytic reduction of the substrate [42].

Thus, it is clear that there is still much to be learned about how a relatively simple switch in loop structures can be manipulated by redox state information contained in two heme cofactors. While bCcPs are one example of such a phenomenon, in the next section we will consider another enzyme, cytochrome cd_1, in which conformational dynamics are also controlled by a multi-heme structure.

2.3. Cytochrome cd_1: redox activation and nitrite reduction

Cytochrome cd_1 nitrite reductase catalyzes the reduction of nitrite to nitric oxide within the denitrification pathway, as well as the four-electron reduction of dioxygen to water [43,44]. Cytochrome cd_1 has been characterized as a heme nitrite reductase, a homodimer with each monomer containing one heme *c* and one heme *d* moiety [44]. The two heme groups are located in the hydrophobic pockets of the two domains. The heme *c* serves as the electron accepting heme and receives electrons in vitro from both cytochrome c_{551} and azurin. Redox titrations have been used to calculate the redox potential of the heme *c* on the order of +250 mV [45]. The heme *d* is the active site heme serving as he binding site for physiological oxidants. The redox potential of the d_1 heme has been estimated to be at least +200 mV or greater [46].

Crystal structures have been solved for both the oxidized and reduced structures of the nitrite reductases from *P. denitrificans* [47–49] and *P. aeruginosa* [50,51]. The domains carrying the d_1 hemes in the oxidized forms of both the *Pd* and *Pa* enzymes are nearly identical, however in the *Pa* enzyme the N-terminal domain, the *c* heme domain, of one monomer crosses the interface between the two monomers and is wrapped around the second monomer [51]. The structural differences between the *Pd* and *Pa* enzymes are all observed in the N-terminal region. In the oxidized state the *Pd c* heme is *bis*-His coordinated, while the *Pa* enzyme *c* heme is His/Met coordinated. In the oxidized *Pd* enzyme the N-terminal arm wraps around the domain of the monomer while the oxidized *Pa* enzyme has an interchanging of the N-terminal arm and wrapping around the second monomer. The reduced enzymes are very similar in conformation, except for the N-terminal arms. Upon reduction the movement of the N-terminal arm, in both the case of the *Pd* and *Pa* enzymes, is required to make the Fe of the d_1 heme available for catalysis [48–52].

Kinetics and thermodynamics of the internal electron transfer process in the *Pseudomonas stutzeri* enzyme have been studied and found to be dominated by pronounced interactions between the *c* and the d_1 hemes [53]. The interactions are expressed both in dramatic changes in the internal electron-transfer rates between these sites and in marked cooperatively in their electron affinity. The results constitute a prime example of intraprotein control of the electron transfer rates by allosteric interactions. The enzyme-reduction state has been analyzed by using a model that involves electron uptake by the *c* hemes followed by equilibration between hemes *c* and d_1 within the same subunit. The model includes only equilibria in which intrasubunit ET can take place [49].

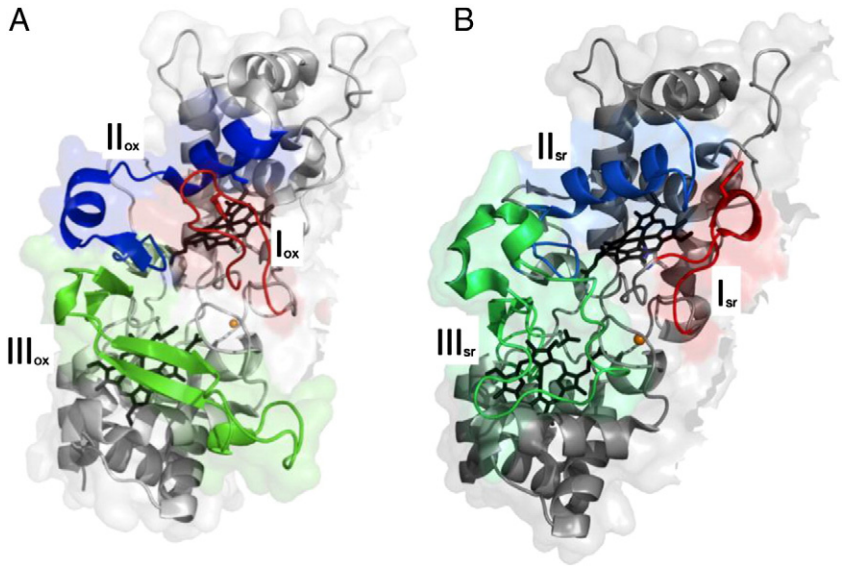

Fig. 2. The conformational differences observed in (A) the fully oxidized form of *Pa* bCcP, and (B) the subsequent reorganization of three loop regions upon chemical preparation of the $Fe_L^{III}Fe_H^{II}$ redox state. (Figure composed using Pymol, with Protein Data Bank files, 1EB7.pdb and 2VHD.pdb (panel B).)

3. Long-range electron transfer: molecular "wires" composed of hemes

3.1. Long range electron transfer

Multiheme cytochromes can possess a variety of functions, including the passage of charge over long distances. In such a function, maintaining appropriate reduction potentials that allow for efficient electron transfer is essential. And indeed, many factors can tune the reduction potential [11]: A heme *c* with bis-histidine ligation has, on average, a midpoint potential range of −400 mV to 0 mV whereas a heme *c* with histidine/methionine ligation has, on average, a higher midpoint potential range of 0 mV to +400 mV [8,11]. Interestingly, while multiheme cytochromes are quite prevalent throughout Nature, there is a notably higher incidence of them in gram negative organisms, where they are localized and matured in the periplasmic space, and thereby linked with respiratory processes with either soluble inorganic, or exogenous electron acceptors (like Fe(III) oxides [13]) as described below.

3.2. Dissimilatory metal reduction

In sedimentary environments, such as aquatic sediments and submerged soils, iron-reducing organisms can grow by coupling the oxidation of organic or inorganic compounds to the reduction of iron in a process referred to as dissimilatory metal reduction (DMR) [54,55]. The DMR process occurs in strictly anaerobic organisms such as *G. sulfurreducens* to aerobic organisms like *P. aeruginosa* to facultative species such as *S. oneidensis* [56]. These species are able to use metal as a terminal electron acceptor, which supports the anaerobic growth of the bacteria. Notably, unlike other cases of metal reduction by organisms, metal uptake is not associated with exogenous metal reduction in the DMR chemistry of *Shewanella* and *Geobacter*. These organisms can reduce a variety of metals like solid iron and manganese oxides [57–59] as well as soluble metals such as uranium and chromium [60,61].

Microbes capable of metal reduction have gained a vast following over the last decade because of their possible use in bioremediation and microbial fuel cells. Bioremediation that involves the capabilities of microorganisms in the removal of pollutants is a promising area of study as it could be a relatively inexpensive means to clean up waste. Additionally, the ability of these organisms to grow biofilms and produce current indicates the possibility that microbial fuel cells could be an alternative source of energy. However, the process by which organisms are able to "breathe" metal is still not entirely understood and the current output from biofilms is relatively small. Thus, investigating the mechanisms of metal reduction and pathways of electron transfer in these organisms is necessary before utilizing them in these enticing applications. Two of the most studied DMR organisms, *G. sulfurreducens* and *S. oneidensis*, have incredibly high cytochrome content, presumably enabling them to use multiple pathways for electron transfer.

Geobacter species are obligate anaerobes found in aquatic sediments. However, the completed genome of *G. sulfurreducens* unveiled its ability to tolerate oxygen [62]. The genome also revealed 111 *c*-type cytochromes, which is higher than what has been reported for any other organism to date [62]. Seventy-three of these contain two or more heme groups, including one that has 27 heme cofactors [62]. The large number of cytochromes suggests that heme-based strategies for electron transport are critical in *Geobacter*. These species can use insoluble Fe(III) and Mn(IV) oxides as terminal electron acceptors [60]. This process is predominantly believed to occur through the use of outer membrane cytochromes [63]. Additionally, they can precipitate uranium in contaminated environments [60]. As such, *Geobacter* is considered an excellent organism for use in bioremediation.

Shewanella species are found in a variety of habitats, from deep sea, anaerobic environments to soil to sedimentary locations, and are exemplified by *S. oneidensis* MR-1. Of its 5000 genes, 42 encode putative *c*-type cytochromes, many of which are directly involved in the bacteria's respiratory pathways that lead to a wide variety of terminal electron acceptors. Unusually, 33 of the 42 *c*-type cytochrome genes in *S. oneidensis* MR-1 contain more than one heme group [64].

This non-pathogenic, gram-negative microbe can grow both aerobically and anaerobically using an extensive assortment of terminal electron acceptors including iron, manganese, uranium, nitrite, nitrate, sulfate, thiosulfate, fumarate, and DMSO in the absence of oxygen [59,61,65–68]. As such, the electron transfer pathway is thought to be highly branched. The anaerobic versatility of *S. oneidensis* is likely a result of its intricate electron-transfer pathway involving many redox-active proteins, include multi-heme *c*-type cytochromes. The redox active

components of this pathway shuttle electrons to terminal electron acceptors, which are reduced during contact with the proteins located on the outer membrane of the bacteria [69,70]. When cells are grown under anaerobic conditions, 80% of the membrane-bound *c*-type cytochrome is localized to the outer membrane. This differs from the results of an aerobic growth in which the cytoplasmic membrane has higher cytochrome content than the outer membrane [71]. These findings suggest that *c*-type cytochromes localized to the outer membrane have a direct role in metal reduction.

Fe(III)-reducing microorganisms are proposed to have developed three different methods by which to transfer electrons from the inside of the cell to extracellular iron: electron shuttling compounds, conductive pili, and finally, direct contact between the ferric iron oxide by *c*-type cytochromes [72]. Fe(III)-reducing microbes have been shown to form biofilms on the surface of iron(III) oxides [73]. Which mechanism is at work may be influenced by the depth of the biofilm and the availability of nutrients [74]. At very shallow biofilm depths, *c*-type cytochromes can make direct contact with the iron oxide, whereas at the middle of the biofilm, electron shuttling compounds may be produced where they can be recycled easily (see below). Finally, at a further distance from the iron oxide surface, conductive nanowires could be at work. At this point it is unclear why organisms have evolved to use these three, possibly energetically unfavorable, mechanisms of dissimilatory metal reduction.

When nutrients are scarce, it is believed that these organisms will reduce Fe(III) minerals through direct contact between outer membrane proteins and the mineral surface [58,75]. In general, cytochromes are not found on the outside of the outer membrane [76]. However, in some DMR organisms, *c*-type cytochromes are oriented on the cell surface, making a direct interaction with iron minerals and reducing them. For instance, there are cytochromes located on the outside of the outer membrane in *S. oneidensis* and *G. sulfurreducens*, which enable direct exchange of electrons with metal oxides [71]. Two *Shewanella* proteins, MtrC and OmcA, are decaheme cytochromes that have been studied extensively over the last decade. Studies have shown that removal of either of the genes encoding these proteins greatly decreases the Mn(IV) and Fe(III) reductase activity [58,75]. Additionally, it has been shown that MtrC and OmcA form a high affinity complex with a heightened ability to reduce Fe(III) [64]. A functional investigation of OmcA showed that it was able to bind and reduce hematite through a direct interaction [77]. In order for this mechanism to be viable, there must be a pathway of electron transfer involving periplasmic proteins to form a respiratory chain to the outer membrane cytochromes, which can then directly contact the iron(III) oxide [71].

3.3. Molecular "wires"

The DMR pathway in *Shewanella* has been studied extensively (see, for example, reference [78]). It is composed of several multiheme cytochromes that form a wire-like pathway from the cell interior to its exterior. Along with *cymA*, the *mtrDEF–omcA–mtrCAB* gene cluster has been shown to participate in DMR chemistry directly. The *mtrDEF* cluster is highly similar to *mtrCAB*, but has been the focus of few specific studies, aside from the recent biochemical and crystallographic characterization of MtrF [79,80]. Electrons are generated in the cytoplasmic membrane, entering the quinol pool, at which point they enter the DMR pathway at CymA, a tetraheme protein anchored in the cytoplasmic membrane, which uses menaquinol-7 as an essential cofactor [81]. From CymA, electrons are transferred to periplasmic proteins such as the decaheme cytochrome MtrA or the tetraheme cytochrome STC, and finally, electrons cross the outer membrane through the beta barrel protein, MtrB to MtrC and OmcA, which are thought to directly interact with iron(III) oxides (shown in Fig. 3). Importantly, redox chemistry appears to proceed from CymA to the other proposed components of the pathway in an iso-potential fashion, where one impact of the redundancy of a large number of hemes, is that there are no obvious thermodynamic barriers or sinkholes to successful electron transfer [82]. The interaction of quinol analogs with CymA appears to do little to affect the potentials of any of the four CymA heme cofactors (−110, −190, −240 and −265 mV), suggesting equal affinity for the oxidized and reduced states [83]. Further, it has been shown recently that beyond the generation of genetic knock-outs, biochemical studies of the individual components of the *Shewanella* Mtr-based pathway of electron transfer demonstrate that electrons can be handed off from one protein to another as shown in Fig. 3, as monitored by biochemistry [84], direct electrochemistry [85] or NMR studies [86]. Electrochemical analyses further illustrate how nature has tuned the proteins of the Mtr pathway to transfer electrons with a sense of kinetic bias, as electrocatalytic currents generated between proteins occur largely in a single direction [85]. That said, it has also been shown that electron transfer from the outside of *Shewanella* to the periplasm is also possible, suggesting the possibility of electrosyntheses conducted via multi-heme electron transfer conduits [87].

In *Geobacter*, OmcB, OmcE, and OmcS have been implicated in extracellular Fe(III) reduction as removal of the genes encoding these proteins results in decreased Fe(III) reduction [63,88]. It is proposed that they will transfer electrons directly to the electrode surface similar to the role of MtrC and OmcA [63,88], but OmcB, OmcE, and OmcS have not been studied to the same extent as the outer membrane cytochromes in *Shewanella*. In both of these organisms, electrons are transferred via a series of heme cofactors that essentially form a molecular wire from the inside of the cell to the outside.

It is also possible to consider the decaheme cytochromes as individual units. For instance, MtrA itself can be thought of as a molecular wire [79,89]. MtrA has a remarkably low amino acid to heme ratio, just 34, whereas an average heme containing protein has 60–70 amino acids per heme cofactor [13]. It may perform the unique function of an intermediary periplasmic protein, transporting electrons from CymA to the outer membrane protein MtrC [85]. A structural characterization of MtrA using small-angle X-ray scattering (SAXS) showed that it possesses a rod-like aspect ratio with a maximum dimension of 104 Å (Fig. 4) [90]. Crystal structures of other multiheme cytochromes have shown that their hemes are packed as a wire with edge-to-edge distances between 4 and 8 Å [91,92]. A similar packing of cofactors in MtrA would suggest that the protein is approximately 100 Å in length and thus able to span a substantial portion of the ~250 Å [93] distance across the periplasm. Overall, these traits are thought to be retained in other paralogs of MtrA, while the other decaheme cytochromes involved in long range electron transfer in *Shewanella* and related organisms (e.g. MtrC, OmcA) are sufficiently divergent to suggest alternative structures [94], as confirmed by the recent structure of MtrF [80] a close paralog of MtrC [95]. Indeed, while MtrA may be thought of best as a wire, the recent X-ray crystal structures of MtrF [80] and an eleven-heme bearing ortholog UndA [96], display a bifurcated cross arrangement, that suggests two intersecting pathways of electron transfer, one that emerges from and is orthogonal to the membrane normal, and another that is parallel to it. While tantalizing, these structures also leave several open questions, including elucidating the nature of interactions with inorganic oxides, redox shuttles, or other proteins. For example it has been established that MtrC can interact tightly with OmcA [58] in *S. oneidensis* in a 2:1 ratio [64], which presumably facilitates successful direct electron transfer with inorganic materials [77].

Unfortunately, the ability to manipulate the chemistry of the multi-heme cytochromes involved in extra-cellular metal reduction has been limited due to the lack of structural data while the majority of the information that is known about these proteins, or in the identification of their orthologs, is derived from bioinformatics studies that rely upon the identification of *c*-type cytochromes via their – CXXCH – heme binding motif. As such, it is hard to identify other types of "nanowire" elements from Nature that may also engage in long-range electron transfer, because they lack a defining amino acid sequence. So while it is possible, and likely, that there are other molecular wires comprised of non-*c*-type hemes, this approach makes it difficult to identify them.

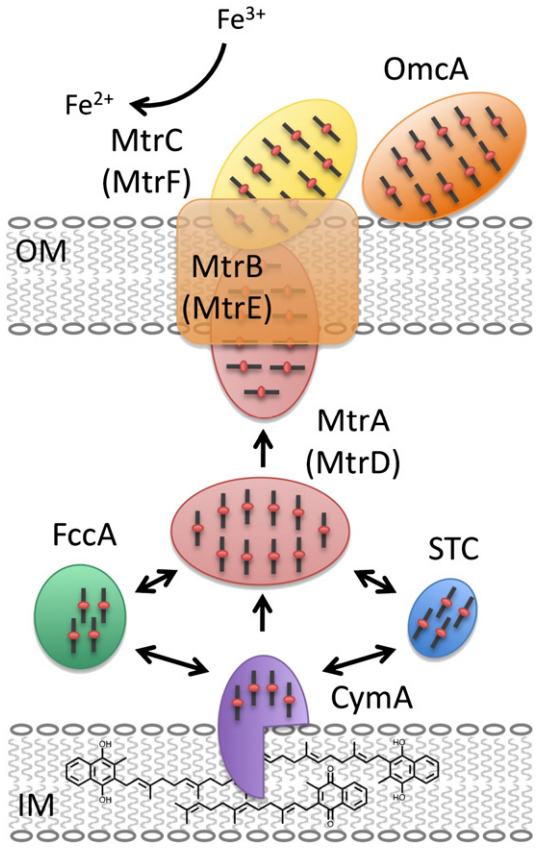

Fig. 3. The dissimilatory metal reduction (DMR) pathway in *Shewanella oneidensis*. A network of multi-heme cytochromes is responsible for the long-range electron transfer from the quinol pool through the tetraheme cytochrome, CymA, across the periplasm via MtrA, and finally to MtrC through the porin protein, MtrB. Arrows indicate substantiated redox reactions through electrochemical and spectroscopic studies (see main text).

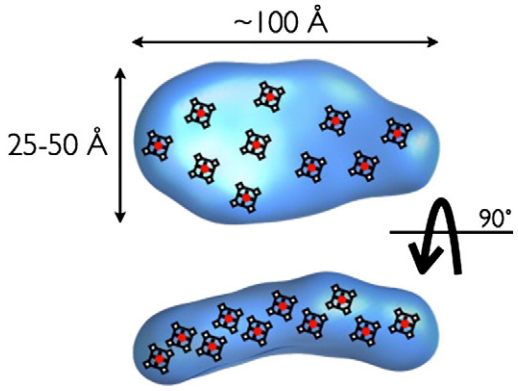

Fig. 4. Depiction of MtrA structure, as described by SAXS, by Firer-Sherwood and co-workers [90], with schematized placement of potential porphyrin structures.

Further, the individual structures that do exist for components of long-range electron transfer pathways often leave more questions than answers. For example, in the case of recent structure of MtrF and its ortholog UndA [96], the potential enzymatic activity, assemblage into larger structures, interaction/recognition of inorganic materials, and ability to participate in redox cycling of flavin compounds (known redox shuttles in *Shewanella* [97,98]), and overall relevance to the similar pathways found in *Geobacter* are all open questions.

4. Storage of reducing equivalents

4.1. Multi-heme enzymes: multi-electron catalysis

Multi-heme containing proteins have the ability to store multiple reducing equivalents [84,99,100], which are useful in Nature and in the biomimetic world. Section 3.3 highlights the ability of multiple neighboring *c*-type hemes to act as a conductive "molecular wire", and here we will discuss how a string of neighboring hemes can be used to store electron equivalents for catalysis, using well illustrated examples of catalysis found in biological nitrification and nitrogen metabolism: cytochrome *c* nitrite reductase (ccNir) and hydroxylamine oxidoreductase (HAO). Intriguingly, the evolutionary relationship between ccNir, HAO and cytochrome c_{554} has been described recently, suggesting a model for an ancient event of truncation that separated the tetraheme proteins from octaheme ancestors, and that pentaheme ccNir proteins resulted from a distinct event of branching from the HAO family [7,12]. The substrate specificities of these enzymes, along with other multi-heme cytochromes *c* have also been recently reviewed [101].

Cytochrome *c* nitrite reductase (ccNir, or NrfA) is a homo-dimeric protein containing five heme groups per monomer [102]. ccNir is able to catalyze the six electron reduction of nitrite to ammonia, the five electron reduction of nitric oxide to ammonia, as well as the two electron reduction of hydroxylamine to ammonia [103]. Hydroxylamine oxidoreductase is a 24 heme containing homo-trimer that catalyzes the four electron oxidation of hydroxylamine to nitrite [92,104–106]. There is a design feature that is shared between these two enzymes: the presence of two pathways for electrons to flow to/from the active site [92,106]. This feature aids in the ability of these enzymes to hold charge generated during catalysis. And, as in the case of bC*c*P enzymes described above, the role of protein quaternary structure may play a role of some significance: ccNir is an obligate dimer, where it has been recently suggested that negative cooperativity between the active sites of the dimer may be due to redox communication between the two protomers [107]; as discussed below, the organization of hydroxylamine oxidoreductase is clearly significant for the organization of the heme units within the protein trimer. The specific example of hydroxylamine oxidoreductase and its partner protein, cytochrome c_{554}, is discussed in further detail below.

4.2. Ammonia oxidation: hydroxylamine oxidoreductase and cytochrome c_{554}

4.2.1. Ammonia oxidation

The enzymatic system responsible for extracting energy from ammonia in *N. europaea* has two catalytic enzymes (ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO)) and two electron transfer proteins (c_{554} and c_{552}) [108]. Ammonia is oxidized to hydroxylamine by AMO, which is subsequently oxidized to nitrite by HAO [109]. Reducing equivalents are shuttled from HAO to c_{554}, c_{552} and ultimately to cytochrome *c* oxidase (Fig. 5). It is interesting to note that HAO, c_{554} and c_{552} have different isoelectric points, which may facilitate the protein–protein interactions necessary for electron transfer. HAO, c_{554} and c_{552} have pI values of 5.3, 10.7 and 3.7 respectively [108,110]. Cytochrome c_{554} has been shown to have a basic patch that is thought to interact directly with HAO [111].

HAO is one of the most complex multi-heme enzymes found in Nature, as the homo-trimer contains 24 heme groups [92,106]. The crystal structure has been solved (Fig. 6A) and shows a complex heme arrangement (Fig. 6B) [92]. The trimer holds 18 of the *bis*-His, *c*-type hemes in a ring, with the catalytic P460 hemes (one per monomer) slightly above the ring. The additional three *c*-type heme groups (one per monomer, Heme 1) are outside of the ring structure. P460 is

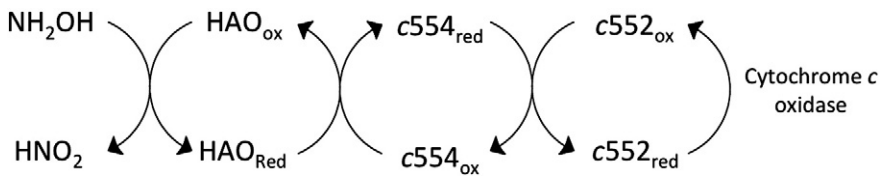

Fig. 5. Ammonia oxidation pathway in *Nitrosomonas europaea*. Hydroxylamine (which is produced from the oxidation of ammonia from ammonia monooxygenase) is oxidized by hydroxylamine oxidoreductase (HAO). Electrons are then transferred through cytochromes c_{554}, c_{552}, and finally to cytochrome *c* oxidase.

a unique heme that is covalently attached to the protein backbone by two cysteine residues (in a typical −CXXCH− motif) plus an unusual tyrosine residue. P460 is pentacoordinated and is the site of hydroxylamine binding.

4.2.2. Hydroxylamine oxidoreductase: electron storage

As mentioned in Section 4.2.1, the heme configuration in HAO is complex and it is this complexity that allows HAO to be an efficient catalyst. Hydroxylamine binds to P460 and the electrons released during the oxidation are quickly transferred from heme to heme in the trimer ring [112]. Recently, the non-equilibrium redox potentials of the hemes were modeled to predict what happens biologically [113]. Previous redox titrations were performed [114,115], yet have not lead to significant insight into the mechanism of electron transfer. However, the results of the spectropotentiometric titrations, do show that all 8 hemes can be reduced [114], further advancing the concept of using HAO as a reducing equivalent storage unit.

The non-equilibrium model predicts that the redox potentials of any given heme group change depending on the oxidation state of the holo-protein and the protein environment (i.e. binding of c_{554}) [113]. This current model suggests that Heme 2 has the highest potential (between +203 mV and +264 mV vs NHE) and is always reduced. The additional electrons will then be able to flow freely through the other hemes, whose redox potentials are modulated to be isopotential, and settle on Hemes 3 and 8. The ring structure allows electron flow between the subunits and supports the "multiple pathway" idea of electron transfer. The solvent exposed Heme 1 has a negative redox potential (−225 mV) and it is the cooperativity of Heme 1 (lowest potential) and Heme 2 (highest potential) that "locks" the electrons within the homo-trimer. HAO's partner protein, c_{554}, is then able to retrieve these electrons by docking near Heme 1, changing the electrostatic environment and increasing the potential of Heme 1, thus releasing its electrons to the hemes in c_{554} [113,116]. This electron transfer has been postulated to be a two-electron transfer. Two electrons are thought to enter c_{554} through Heme II and the subsequent intramolecular electron transfer to Heme I is fast, where the two hemes operate as a single redox unit. Stopped-flow experiments have shown that HAO and c_{554} do exchange electrons in vitro: Two electrons have been found to be transferred, each at a different rate: the first at a rate of 250–300 s^{-1} and the second at a rate of 25–30 s^{-1} [116].

Spectroelectrochemical analysis of cytochrome c_{554} revealed the presence of three unique midpoint potentials: +47, −147, and −276 mV (vs NHE) [117,118], which are depicted in Fig. 7A. Further analysis using a coulometric titration showed that the high potential couple was associated with two electrons stoichiometrically, thus accounting for all four redox active heme groups [117]. Protein film voltammetry (PFV) experiments have further resolved the midpoint potentials and the number of electrons in each couple [119]. Hemes I to IV are found to have potentials of +32, +50, −183 and −283 mV, respectively. Additionally, the cooperativity between hemes I and II was observed from direct electrochemistry (Fig. 8), as an apparent "narrowing" of the voltammograms associated with the Heme I/II couples. Such behavior verifies the observation of a desirable trait in many bioenergetics systems: the ability to selectively deliver or accept multiple redox equivalents. Fig. 8 further illustrates the challenge associated with observing such cooperativity: here the crossing of the redox potentials results in narrowing of the electrochemical signatures, but the effect is subtle. Clearly such phenomena may be lost in cases where there are more than two redox couples in close proximity, such as the cases of the *Shewanella* cytochromes discussed above [82,79], or ccNiR [120,121]: in all of these cases, heme redox potentials appear to be closely spaced in terms of overall

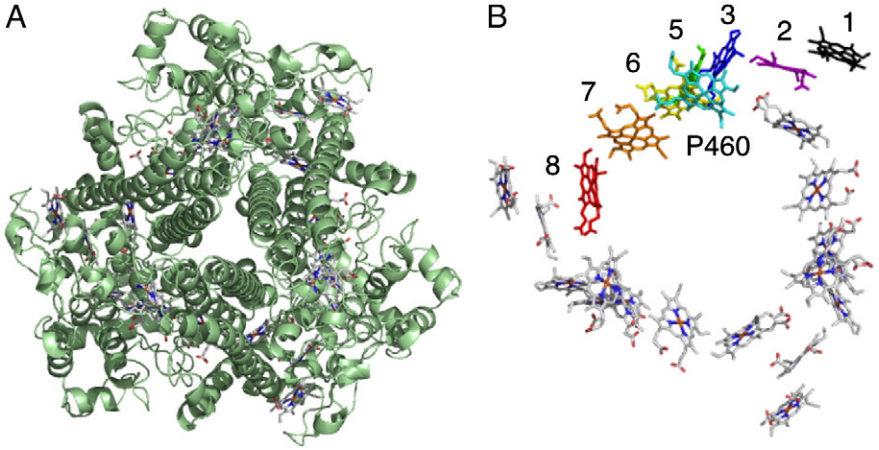

Fig. 6. Hydroxylamine oxidoreductase (HAO) is a homotrimer containing 24 heme groups (A). Each monomer contains 8 heme groups with a unique P460 heme (shown in cyan, B). Figure constructed from Protein Data Bank file 1FGJ.pdb.

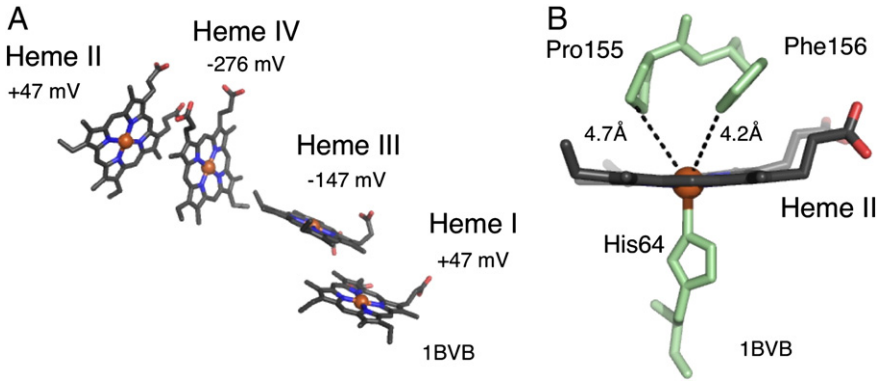

Fig. 7. Heme configuration of c_{554} shows two sets of parallel heme groups (A). The high potential heme groups (Hemes I and II) are the furthest apart, spatially (28.1 Å, Fe–Fe). The heme groups are *bis*-His ligated, except for Heme II. Heme II is penta-coordinated with one ligated histidine (B). (Protein Data Bank entry 1BVB.pdb.)

thermodynamics, and no cooperativity has been reported to date. The molecular basis for redox cooperativity is poorly understood, and may arise in part from fast intramolecular electron transfer (the hypothesized ET route is from hemes II to IV to III to I [118,122]). This intramolecular electron transfer appears to be much faster than intermolecular or interfacial ET [119]. The coupling of hemes I and II along with observed gating may partially support the strict two-electron ET observed in nature between HAO and c_{554} [116,119].

N. europaea has optimized this ammonia oxidation system in vivo. When taken out of context, it has been found that HAO is not quite an efficient machine, as side reactions have been observed, including its ability to run in reverse and be a cytochrome *c* nitrite reductase [123]. HAO functions differently in the presence or absence of oxygen. The oxidation of hydroxylamine by HAO occurs aerobically. Anaerobically, HAO has the ability to reduce nitric oxide or hydroxylamine [124], at least in vitro. This leaky system poses challenges to both in vitro studies and its potential off-label utilization as an electron storage unit.

4.2.3. Cytochrome c_{554}: alternative function

Some researchers have speculated that c_{554} may have another function within the cell; that it may moonlight as an NO reductase. Joint work from the Hendrich Laboratory (Carnegie Mellon University) and the Hooper Laboratory (University of Minnesota) has shown that Heme II from c_{554} has the ability to bind and reduce nitric oxide [125].

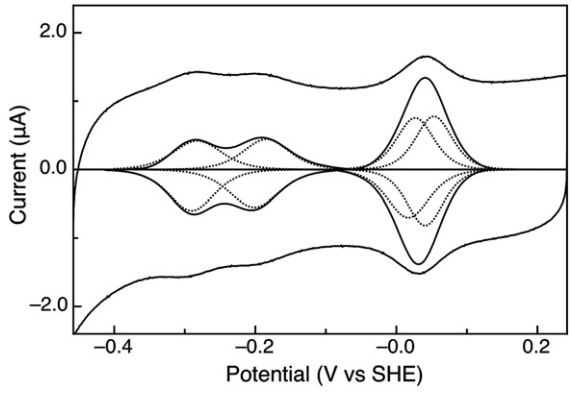

Fig. 8. A cyclic voltammogram of c_{554} on 4-mercaptobenzoic acid modified gold (scan rate of 1 V, 4 °C, pH 7); both raw and baseline subtracted data. The four heme groups are modeled in with dashed lines.

The crystal structure of both oxidized and fully reduced c_{554} has been solved. The overall fold is predominately alpha helical, and the four heme groups are orientated in two sets of parallel molecules (Fig. 7A). The numbering of the hemes is from the order in which they appear in the sequence. Heme groups I, III and IV are *bis*-His ligated, while Heme II is pentacoordinated (one His ligand) (Fig. 7B). From the structure alone it is difficult to conjecture why Heme II has an open coordination site (Fig. 7). It is open in the sense that there is not a sixth ligand, yet Pro155 and Phe156 are in close proximity [111]; either blocking access completely or adding specificity to which small molecules can bind. One challenge is to determine if this penta-coordinate heme is an advantageous product of evolution, or if it is an accidental artifact of the evolutionary process. However it arose, this penta-coordinate site could be exploited for new reactivity. It is not unrealistic to think that the coordination sphere can be tuned in such a way to make c_{554} a designer catalyst.

5. Concluding remarks: multi-heme proteins as molecular tools

The traits of multi-heme proteins highlighted in this review (redox linked conformational changes, the capacity to yield vectorial electron transfer over the length scale of 10^2 Å, and the ability to store and manipulate $n > 1$ redox equivalents) all point the potential utility multi-heme proteins may provide to the emerging field of synthetic biology, where either multi-electron redox catalysis, long-range electron transfer or redox-based conformational dynamics may be required as a component within a novel biological process. For example, the possibility of using reverse electron flow to achieve electrosynthesis in *S. oneidensis* via electron transfer *into* the cellular interior has already been realized [73], and seems an attractive approach to achieve electrosyntheses in other host organisms. At the same time, several challenges remain in the ability to rationalize the electronic and chemical properties of multi-heme cytochrome *c*, whether as enzymes or as electron transfer elements. For example, while the ability to incorporate the MtrCAB complex from *S. oneidensis* into *Escherichia coli* to provide for an exogenous redox conduit has been demonstrated [126], a way forward to improve upon the multi-heme electron transfer complex, by design, is still lacking in our attempts to improve upon Nature's apparent mastery.

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