On the road to improve the bioremediation and electricity-harvesting skills of Geobacter sulfurreducens: functional and structural characterization of multihaem cytochromes

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Abstract
Extracellular electron transfer is one of the physiological hallmarks of Geobacter sulfurreducens, allowing these bacteria to reduce toxic and/or radioactive metals and grow on electrode surfaces. Aiming to functionally optimize the respiratory electron-transfer chains, such properties can be explored through genetically engineered strains. Geobacter species comprise a large number of different multihaem c-type cytochromes involved in the extracellular electron-transfer pathways. The functional characterization of multihaem proteins is particularly complex because of the coexistence of several microstates in solution, connecting the fully reduced and oxidized states. NMR spectroscopy has been used to monitor the stepwise oxidation of each individual haem and thus to obtain information on each microstate. For the structural study of these proteins, a cost-effective isotopic labelling of the protein polypeptide chains was combined with the comparative analysis of \(^{1}H\)-\(^{13}C\) HSQC (heteronuclear single-quantum correlation) NMR spectra obtained for labelled and unlabelled samples. These new methodological approaches allowed us to study G. sulfurreducens haem proteins functionally and structurally, revealing functional mechanisms and key residues involved in their electron-transfer capabilities. Such advances can now be applied to the design of engineered haem proteins to improve the bioremediation and electricity-harvesting skills of G. sulfurreducens.

Geobacter species as a biotechnological target
The first Geobacter strain described in the literature was isolated in 1987 from freshwater sediments. Since then, numerous physiological and genetic studies have revealed the respiratory versatility and environmental relevance of Geobacter species in natural habitats, as well as its potential for applications in the field of bioenergy production and bioremediation [1]. Examples of such applications include (i) degradation of hydrocarbon contaminants in soils, (ii) reduction of insoluble Fe(III) and Mn(IV) oxides, (iii) precipitation of uranium in contaminated aquifers, and (iv) electron transfer on to electrodes, from which electricity can be harvested [2–4]. One conceivable route to improve the use of Geobacter species in such biotechnological applications is the optimization of key proteins mediating electron transfer in their respiratory chain(s). Several c-type cytochromes, most of them containing more than one haem group, have been shown to be required for growth in the presence of different electron acceptors and might explain the respiratory versatility presented by Geobacter [5–15].

In the present review, recent methodological improvements and their applications towards the characterization of Geobacter sulfurreducens multihaem cytochromes in solution are revisited.

Multihaem cytochromes involved in extracellular electron transfer
G. sulfurreducens was the first Geobacter species for which methods for genetic manipulation were developed; this is the reason it has been used as a model for the study of Geobacteraceae family [16,17]. Many of the terminal electron acceptors used by G. sulfurreducens are insoluble and thus unable to diffuse into the cells. Consequently, the reduction of these acceptors requires the transfer of electrons across the outer membrane to the cell exterior [18,19]. This is assisted by an unusual cellular topology of the electron-transfer proteins in G. sulfurreducens cells when compared with other micro-organisms. In fact, in addition to the common location in the inner membrane and periplasmic space, electron-transfer components have also been discovered in the outer membrane [6]. Gene-knockout and proteomic studies have led to the identification of putative periplasmic and outer membrane multihaem c-type cytochromes involved in extracellular electron transfer (Table 1). On the basis of these observations, a model for...
Table 1 | Summary of gene-knockout and proteomic studies on *G. sulfurreducens* c-type cytochromes

Mac, membrane-associated cytochrome; Omc, outer-membrane cytochrome; Pgc, periplasmic GEMM-regulated cytochrome; Ppc, periplasmic cytochrome.

<table>
<thead>
<tr>
<th>Protein (GSU number)</th>
<th>Predicted number of haem groups</th>
<th>Predicted cellular localization</th>
<th>Gene knockout and proteomic studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>PpcB (GSU0364)</td>
<td>3</td>
<td>Periplasm</td>
<td>Double deletion mutant with PpcC has U(VI) reduction capability affected [9]. Detected in both Fe(III) citrate and Fe(III) oxide cultures, but more abundant in Fe(III) citrate [48]</td>
</tr>
<tr>
<td>PpcC (GSU0365)</td>
<td>3</td>
<td>Periplasm</td>
<td>Double deletion mutant with PpcB has U(VI) reduction capability affected [9]</td>
</tr>
<tr>
<td>MacA (GSU0466)</td>
<td>2</td>
<td>Periplasm</td>
<td>In the deletion mutant: reduction of U(VI) is affected [9]; growth on Fe(III) citrate is impaired, by affecting OmcB expression [10]. More abundant during growth with Fe(III) oxides compared with Fe(III) citrate as electron acceptor [48]</td>
</tr>
<tr>
<td>PpcA (GSU0612)</td>
<td>3</td>
<td>Periplasm</td>
<td>In the deletion mutant: U(VI) and AQDS reduction is affected when acetate is the electron donor [41]. Fe(III) oxides reduction is affected [9]. Detected in both Fe(III) citrate and Fe(III) oxide cultures [48]</td>
</tr>
<tr>
<td>GSU0616</td>
<td>8</td>
<td>Periplasm</td>
<td>Deletion mutant has increased Fe(III)- and U(VI)-reducing activity [9]</td>
</tr>
<tr>
<td>OmcE (GSU0618)</td>
<td>4</td>
<td>Outer membrane</td>
<td>In the deletion mutant: growth in Fe(III) and Mn(IV) oxides is affected [6]. Current production is affected, but adapts with time [7]; reduction of U(VI) is affected [9]. No impact on current production [12]. More abundant during growth with Fe(III) citrate compared with fumarate as electron acceptor [40]</td>
</tr>
<tr>
<td>PpcD (GSU1024)</td>
<td>3</td>
<td>Periplasm</td>
<td>More abundant during growth with Fe(III) oxides compared with Fe(III) citrate as electron acceptor [48]</td>
</tr>
<tr>
<td>GSU1334</td>
<td>7</td>
<td>Outer membrane</td>
<td>In the deletion mutant: reduction of U(VI) and Fe(III) oxides is affected [9]. More abundant during growth with Fe(III) oxides compared with Fe(III) citrate as electron acceptor [48]</td>
</tr>
<tr>
<td>PpcE (GSU1760)</td>
<td>3</td>
<td>Periplasm</td>
<td>Found only in cultures with Fe(III) citrate [48]</td>
</tr>
<tr>
<td>PgcA (GSU1761)</td>
<td>3</td>
<td>Periplasm</td>
<td>More abundant during growth with Fe(III) oxides compared with Fe(III) citrate as electron acceptor [48]. Increased expression in strains adapted to grow in Fe(III) oxides [15]</td>
</tr>
<tr>
<td>OmcZ (GSU2076)</td>
<td>7</td>
<td>Outer membrane</td>
<td>Deletion mutant shows severely inhibited current production and biofilm formation [12]</td>
</tr>
<tr>
<td>OmcF (GSU2432)</td>
<td>1</td>
<td>Outer membrane</td>
<td>In the deletion mutant: growth on Fe(III) citrate is impaired, by affecting OmcB expression [5]; reduction of U(VI) is affected [9]; there is decreased current production [11]</td>
</tr>
<tr>
<td>OmcS (GSU2504)</td>
<td>6</td>
<td>Outer membrane</td>
<td>In the deletion mutant: growth in Fe(III) and Mn(IV) oxides is affected [6]. There is no impact on current production [12]. Localized along the pili [49]</td>
</tr>
<tr>
<td>OmcC (GSU2731)</td>
<td>12</td>
<td>Outer membrane</td>
<td>Fe(III) [50] and U(VI) [9] reduction not affected in the deletion mutant</td>
</tr>
<tr>
<td>OmcB (GSU2737)</td>
<td>12</td>
<td>Outer membrane</td>
<td>Deletion mutant grows poorly in Fe(III) citrate and does not grow in Fe(III) oxides [50]; there is no impact on U(VI) reduction [9], electrode reduction [7] and current production [12]. Affected by deletion of omcF [5], macA [10], omcC and omcH genes [8]. More abundant during growth with Fe(III) citrate compared with fumarate as electron acceptor [40]</td>
</tr>
<tr>
<td>GSU3332</td>
<td>2</td>
<td>Outer membrane</td>
<td>Reduction of U(VI) and Fe(III) oxides is affected in the deletion mutant [9]</td>
</tr>
</tbody>
</table>

electron transfer to Fe(III) oxides in *G. sulfurreducens* has been proposed (Figure 1). To validate this model and gain deeper insights into the mechanisms of extracellular electron transfer, structural and functional studies of the proteins involved are essential. Unfortunately, the characterization of multihaem cytochromes is not a straightforward task as considerable difficulties arise at both the structural and the functional level.

**Inherent difficulties in multihaem cytochrome characterization**

**Macroscopic compared with microscopic redox characterization**

The presence of several haem groups in cytochromes constitutes the first obstacle in their characterization. Indeed, in monohaem cytochromes, only the fully reduced and
Electron Transfer at the Microbe–Mineral Interface

**Figure 1 | Proposed model depicting the extracellular electron-transfer pathway to Fe(III) oxides by *G. sulfurreducens***

The model is based on previous models [3,19] and subsequent findings [15,49] (see also Table 1). The white path represents the proposed electron-transfer pathway. The membrane-associated cytochrome MacA receives electrons from the menaquinol (MQH$_2$)/menaquinone (MQ) pool at the inner membrane and reduces the periplasmic trihaem cytochromes (PpcA–PpcE). These cytochromes mediate the electron transfer from the periplasm to the outer-membrane-associated cytochromes (OmcB, OmcE and OmcS) that are likely to be directly involved in the reduction of insoluble Fe(III) oxides. OmcS was shown to be localized along the pili when *G. sulfurreducens* grows in Fe(III) oxides. The midpoint reduction potential of the individual haems of the periplasmic c$_7$ cytochrome family, except for PpcC, have been determined and cover the redox potential range $-162$ to $-100$ mV [20]. For OmcS only, the macroscopic reduction potential was determined and is $-212$ mV [14].

oxidized states may coexist in solution, whereas in multihaem cytochromes, several one-electron reversible transfer steps convert the fully reduced state into the fully oxidized state (Supplementary Figure S1 at http://www.biochemsoctrans.org/bst/040/bst0401295add.htm). Consequently, different redox stages are defined, each grouping microstates with the same number of oxidized haems [20]. Therefore the redox potentials measured by voltammetry or visible redox titrations only describe macroscopically the redox behaviour of the protein [13,14,21,22]. Although providing information on the working functional ranges of the proteins, in most cases, the macroscopic redox potentials are insufficient to give mechanistic information on the electron-transfer pathways. This can only be achieved when the fractional contribution of each possible microstate during the oxidation of the protein is obtained.

In multihaem cytochromes containing neighbouring haem groups, the reduction potential of each haem is most often modulated by redox interactions with other haems and by the solution pH (redox-Bohr interactions). Thus, to completely characterize the redox centres of a multihaem cytochrome, it is necessary to determine the haem-reduction potentials, the redox interactions and the properties of the redox-Bohr centre(s). To determine these thermodynamic parameters, each haem-oxidation profile at several pH values by two-dimensional EXSY (exchange NMR spectroscopy) has to be monitored. This can only be achieved in conditions of fast intramolecular electron exchange (between the different microstates within the same oxidation stage) and slow intermolecular electron exchange (between different oxidation stages) on the NMR timescale [23]. Under such conditions, the haem-oxidation fraction can be determined from the chemical shifts of the haem substituents in the different oxidation stages. Haem methyl resonances are the most identifiable NMR signals of all haem substituents, making them the ideal candidates to follow the stepwise oxidation of the haems throughout a redox titration. The paramagnetic shifts of the haem methyls are proportional to the oxidized fraction of a particular haem and, as a result, contain information about the redox properties of each haem group [20]. However, the NMR data are insufficient to determine the absolute thermodynamic
parameters and need to be complemented with data from redox titrations monitored by visible spectroscopy [24]. Once the thermodynamic parameters are determined, it is possible to evaluate the contribution of each microstate and their relevance to the electron-transfer mechanism.

**Solution structure determination**

Gathering structural information on multihaem cytochromes is crucial to understand their functional mechanisms. However, the determination of the structures in solution is also a bottleneck in the characterization of multihaem cytochromes and explains the shortage of solution structures described in the literature ([25,26], and references therein). This is undoubtedly associated with the poor expression yields obtained for mature proteins (<1 mg/l of culture), that renders their isotopic labelling unproductive, and with the inherent complex analysis of the NMR spectra. Additionally, the assignment of the NMR signals is complicated further by the presence of numerous proton-containing groups in the haem groups and the magnetic properties of the haem iron, particularly in the oxidized state [26]. The number of NOE (nuclear Overhauser effect) connectivities involving the haem protons is substantially higher compared with those of each amino acid and, for that reason, their assignment cannot be neglected or discarded in the structure determination ([25,26], and references therein). Until recently, crystal structures had been reported for *G. sulfurreducens* multihaem cytochromes only [21,27–32]. NMR spectroscopy enables us to go beyond this static characterization with the determination of structures proteins in similar conditions close to their physiological environment, providing information about the protein internal motions and interactions in solution.

**Recent advances in multihaem cytochrome characterization**

In the last decade, several experimental and technological advances opened new perspectives to the study of multihaem proteins. These encompass improvements in the protein expression protocols, as well as in the equipment and NMR technology.

The development of an efficient expression system to produce multihaem c-type cytochromes using *Escherichia coli* as a host was successfully applied to the expression of cytochromes containing up to 12 haem groups [27,33–35]. We then showed that this expression system could be used to achieve cost-effective labelling of multihaem cytochromes; a crucial step to reduce the time required to assign the protein NMR signals [36].

Most recently, using the trihaem cytochrome PpcA from *G. sulfurreducens* as a model, we presented a strategy that simplifies the assignment of the NMR signals in multihaem proteins and, concomitantly, their solution structure determination [26]. This strategy combined the analysis of *1H-13C* HSQC (heteronuclear single-quantum coherence) NMR spectrum obtained for PpcA labelled in its polypeptide chain with spectrum obtained for unlabelled protein, allowing the straightforward discrimination between the haem groups and the polypeptide chain signals (Figure 2). This is the latter landmark for gathering structural data in solution for multihaem cytochromes [26,37–39] and new strategies towards the investigation of these proteins can now be developed.

The characterization of *G. sulfurreducens* multihaem cytochromes in solution is still in the early stage, but considerable progresses have been made using the new methodological strategies described above. In multihaem cytochromes, the use of high-field magnetic NMR spectrometers equipped with cryoprobes favours the slow intermolecular electron-exchange regime between different oxidation stages, allowing us to monitor the stepwise oxidation of each haem [23]. Data obtained from NMR experiments carried out on these spectrometers equipped with cryoprobes and from redox titrations monitored by visible spectroscopy, allowed us to study the redox properties of four periplasmic trihaem cytochromes (also known as c7 cytochromes) and of one domain of the 12-haem cytochrome from *G. sulfurreducens* at the microscopic level.

The family of c7 cytochrome from *G. sulfurreducens* is considered essential in the bacterial electron-transfer pathways, since soluble periplasmic cytochromes are the likely reservoir of electrons destined for the outer surface [3,40,41]. This family comprises five cytochromes, namely PpcA and its homologues (PpcB, PpcC, PpcD and PpcE), of approximately 10 kDa each. The PpcA homologues share 77, 62, 57 and 65% amino acid sequence identity respectively with PpcA [28]. The proteins’ core is composed by the three haem groups covalently linked to the cysteine residues of the CXXCH-binding motifs, with all of the haems axially co-ordinated by two histidine residues. The spatial arrangement of c7 cytochromes is superimposable with that of the structurally homologous tetrahaem cytochromes c1, with the sole difference being the absence of the haem II and the correspondent polypeptide segment. For this reason, the three haem groups in cytochromes c7 are numbered I, III and IV [42].

The haem core structures are similar, with haems I and IV roughly parallel to each other and with both of them nearly perpendicular to haem III [23,28,30]. At the N-terminus, all of the structures have a conserved two-strand β-sheet that is followed by different helical regions in each protein [23,28,30]. The main conserved region is the positively charged surface around haem IV and the lowest similarity is found near haem I [30].

With the exception of PpcC, for which the presence of different conformations in solution impaired its full characterization [43], the results obtained for the other four cytochromes c7 showed that the haem reduction potentials are negative and different from each other with distinct functional working ranges [20,44]. These reduction potentials are strongly modulated by redox and redox-Bohr interactions, establishing dissimilar co-operative networks for each protein. PpcA and PpcD appear to be optimized to interact with specific redox partners, each
Figure 2 | Two-dimensional $^1$H-$^{13}$C HSQC NMR spectra of oxidized PpcA: isotopically labelled ($^{13}$C/$^{15}$N) in its polypeptide chain (black contours) and unlabelled (red contours). Spectra were acquired at 25°C. In both samples, the haem groups are unlabelled. Red labels indicate the haem substituent signals. The signals of protons connected to the same carbon atom (CH$_2$ groups) are linked by a straight line. The ribbon diagram of PpcA reduced structure (PDB code 2LDO) [46] is indicated in the inset. The peptide chain and the haems are coloured black and red respectively. Residues involved in pH-dependent conformational changes (green) and in the putative interacting region with other molecules (blue) are indicated on the PpcA structure.

involving e$^-$/H$^+$ transfer through different mechanisms (Supplementary Figure S1). No evidence for a preferential electron-transfer pathway or e$^-$/H$^+$ coupling was found for PpcB or PpcE in the physiological pH range.

In addition to the trihaem periplasmic cytochromes from G. sulfurreducens, 18 $c_7$-type cytochromes were also found in other deltaproteobacteria: Geobacter metallireducens (five cytochromes), Geobacter uraniireducens (four cytochromes), Geobacter bemidjiensis (three cytochromes), Anaeromyxobacter dehalogenans and Geobacter lovleyi (two cytochromes each), and Desulfuromonas acetoxidans strain 5071 and Pelobacter propionicus (one cytochrome each) [45]. The sequence alignment of these proteins showed that out of 21 highly conserved residues, only nine are not cysteine or histidine residues involved directly in haem binding [45]. Among them, a single aromatic residue is conserved in all of the proteins: Phe$_{15}$. This residue is part of a conserved structural arrangement involving haems I and III. An equivalent arrangement is also observed in tetrahaem cytochromes $c_3$ [42], as well as in cytochromes with $c_7$-type domains [29]. To elucidate the physiological role of Phe$_{15}$, this residue was replaced by leucine using site-directed mutagenesis [45]. In this mutant, the redox properties of the haem groups were determined and compared with those of the wild-type. Not only was the balance of the global network of co-operativities altered, but also the concerted e$^-$/H$^+$ transfer was disrupted. To investigate the physiological role of other PpcA key residues, a family of several different mutants was prepared by site-directed mutagenesis. Their characterization showed that the functional mechanism of PpcA relies on a fine-tuned balance of redox and redox-Bohr interactions, assuring a coherent electron-transfer pathway coupled to proton transfer (L. Morgado and C.A. Salgueiro, unpublished work).
In order to structurally rationalize the redox properties obtained and map the interacting regions with physiological partners, it is fundamental to obtain structural data in solution. The expression system developed by Londer et al. [35] was applied in the isotopic labelling of PpcA [26,36], and NMR spectroscopy was used to determine the solution structure of this protein, its backbone dynamics and the pH-dependent conformational changes [46]. The redox-Bohr centre responsible for controlling the e⁻/H⁺ transfer was identified, as well as the putative interacting regions between PpcA and its redox partners (Figure 2). The solution structures of the other *G. sulfurreducens* c₂ cytochromes are currently being determined. PpcA structure was the first solution structure described in the literature for a *G. sulfurreducens* cytochrome and constitutes one important foundation to the rational understanding of the electron-transfer mechanism of *G. sulfurreducens* respiratory chains.

Another example of a multihaem cytochrome from *G. sulfurreducens* is the dodecahaem cytochrome GSU1996, which is arranged in a ‘crescent-shaped’ architecture and has been characterized as a protein ‘nanowire’ of haems that may work like an electron-storage sink or capacitor at the bacterial periplasm. It is organized into four domains (A–D), each containing three haems with homology with cytochromes c₂, with the difference being haem IV presenting histidine–methionine axial co-ordination [29]. The redox properties of domain C have been determined and showed that the reduction potentials of the haems in the fully reduced and protonated protein are negative and strongly modulated by redox interactions [47]. The significant redox interactions between the haems extend the range of their operating potentials. This suggests that the presence of redox centres closely and strategically arranged along the polypeptide chain constitutes an important advantage spanning the protein redox functional range and probably allowing the bacterium to use different electron acceptors. Studies are in progress to characterize the other domains of GSU1996.

**Future perspectives**

The recent methodological advances described in the present review, together with the emergent availability of genetic systems for *Geobacter*, are expected to shed light on the physiological function(s) of multihaem cytochromes and on the architecture of the respiratory chain(s) of these microorganisms. The cost-effective isotopic labelling of multihaem cytochromes will contribute to increase the number of solution structures in databases. In addition, the greater availability of backbone and side-chain resonance maps can be explored to study the protein dynamic properties, to identify redox- or pH-linked conformational changes and the interacting regions between redox partners. This information can then be used in the rational design of mutated multihaem cytochromes, an essential step towards engineering strains of *Geobacter* or other species with increased respiratory rates and with the concomitant improvement of their biotechnological applications, including microbial electricity production.

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SUPPLEMENTARY ONLINE DATA

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Figure S1 | Electronic distribution scheme for a trihaem cytochrome with a proton-linked equilibrium

In the left-hand panel the 16 possible microstates are represented. The inner circles represent the haem groups, which can be reduced (black) or oxidized (white). Continuous and broken lines in the outer circles represent the protonated and deprotonated microstates respectively. The microstates are grouped according to the number of oxidized haems in four oxidation stages (S0–S3) connected by one electron step. $P_{0H}$ and $P_{0}$ represent the reduced protonated and deprotonated microstates respectively. $P_{ijkH}$ and $P_{ijk}$ indicate the protonated and deprotonated microstates respectively, where $i$, $j$, and $k$ represent the haem(s) that are oxidized in that particular microstate. In the right-hand panel, the dominant microstates in solution for PpcA (upper) and PpcD (lower) are highlighted.