The roles of CymA in support of the respiratory flexibility of *Shewanella oneidensis* MR-1

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Abstract

*Shewanella* species are isolated from the oxic/anoxic regions of seawater and aquatic sediments where redox conditions fluctuate in time and space. Colonization of these environments is by virtue of flexible respiratory chains, many of which are notable for the ability to reduce extracellular substrates including the Fe(III) and Mn(IV) contained in oxide and phyllosilicate minerals. *Shewanella oneidensis* MR-1 serves as a model organism to consider the biochemical basis of this flexibility. In the present paper, we summarize the various systems that serve to branch the respiratory chain of *S. oneidensis* MR-1 in order that electrons from quinol oxidation can be delivered the various terminal electron acceptors able to support aerobic and anaerobic growth. This serves to highlight several unanswered questions relating to the regulation of respiratory electron transport in *Shewanella* and the central role(s) of the tetrahaem-containing quinol dehydrogenase CymA in that process.

Introduction

The gammaproteobacterium *Shewanella oneidensis* MR-1 colonizes various changeable marine and freshwater environments. This capability is underpinned by a flexible respiratory system able to couple the oxidation of molecules such as lactate, pyruvate, protein and DNA to the reduction of a range of terminal electron acceptors located either within or outside the cell (Table 1). These electron-transfer events release free energy that can be harnessed in the form of a pmf (proton-motive force) across the microbial inner membrane. The pmf has both a chemical (ΔpH) and an electrical (Δψ) component, and it is directly responsible for driving ATP synthesis.

Whichever combination of respiratory electron donor and acceptor contribute to maintaining the pmf, a key component of the electron-transfer chain is the redox cycling of Qs (quinones) [1]. Qs are diffusible lipophilic molecules that are confined to the microbial inner membrane where they are reduced to QH2s (quinols) by the reversible addition of two electrons and two protons. During aerobic and microaerobic growth, O2 serves as terminal electron acceptor with the immediate electron donor to the Q-pool being respectively NADH, via the action of NADH-Q oxidoreductases, and formate, through the action of formate dehydrogenases [2]. Several enzymes then allow QH2 oxidation to be coupled to O2 reduction (Table 1). The result is a branched electron-transport chain in which the pmf that is generated per QH2 molecule oxidized can be varied to meet physiological need.

Three terminal oxidases that catalyse the respiratory reduction of O2 have been identified in *S. oneidensis* MR-1. Cytochrome *bd* contributes to the pmf without being a proton pump by virtue of its ability to oxidize QH2 and deliver the two released protons to the periplasm. It is likely to operate as a high-affinity oxidase at low O2 tensions or under growth conditions that may be considered stressful to *S. oneidensis* MR-1. The cytochrome *aa3-* and *cbb3-*type oxidases are proton pumps likely to operate under O2-replete and -depleted conditions respectively. These terminal oxidases receive electrons from the action of the QH2-oxidizing cytochrome *bc1* complex via cytochrome *c*. Cytochrome *bc1* contributes to maintaining the pmf through the Q-cycle, so six protons are translocated across the inner membrane per QH2 oxidized when the cytochrome *aa3-* and *cbb3-*type enzymes reduce O2 [3].

CymA: a constitutive QH2 dehydrogenase

Another QH2 dehydrogenase present during aerobic and microaerobic growth of *S. oneidensis* MR-1 is CymA [4]. This enzyme is a member of the NapC/NirT family of QH2 dehydrogenases. It possesses a single-transmembrane α-helix and a periplasmic globular domain containing four c-type haems. Magnetic circular dichroism has established that three of these haems have histidine–histidine axial ligation [5]. The fourth haem has histidine–water axial ligation and forms an intrinsic part of the QH2-oxidation site. Spectrophotoelectrometric titration has defined midpoint potentials (E°') at pH 7 of approximately −110, −190 and
-- 265 mV for the three low-spin haems and approximately -- 240 mV for the high-spin haem; all potentials are quoted compared with the SHE (standard hydrogen electrode). These E° values are consistent with the envelopes of reductive and oxidative current visualized by cyclic voltammetry of CymA adsorbed on gold electrodes [6]. Similar behaviour is seen for CymA adsorbed on graphite electrodes (Figure 1).

To investigate whether CymA reacts with O2, an aliquot of air-equilibrated buffer was introduced into the voltammetric experiment (Figure 1). This resulted in the appearance of a clear catalytic reduction wave where the onset of catalysis at approximately --100 mV coincides with reduction of the haem cofactors. The catalytic currents are also observed at potentials that are significantly higher than required to observe the direct reduction of O2 by the electrode at a comparable rate (Figure 1). Thus CymA can reduce O2. Whether this reaction occurs in vivo and, if it does, the nature of its contribution to the physiological demands of the cell remain to be established. In vitro experiments have demonstrated electron transfer from CymA to the cytochrome c peroxidase CcpA via monohaem cytochrome ScyA [7]. CcpA removes potentially harmful peroxides and hydroxide radicals produced in the periplasm as unwanted products of O2 reduction. Electron transfer from CymA to CcpA may afford S. oneidensis MR-1 protection against reactive oxygen species produced in fluctuating oxygen levels and perhaps allow peroxide to serve as a terminal electron acceptor.

CymA is expressed constitutively, but expression can increase during anaerobic growth where, in contrast with the situation in aerobic growth, the physiological contributions of CymA are well established. CymA serves as a QH2 dehydrogenase that is able to supply electrons to numerous protein and enzyme systems dedicated to reducing a variety of terminal electron acceptors (Table 1). Deletion of cymA has shown that the gene product is essential for the terminal reduction of fumarate, nitrate, nitrite, soluble complexes of Fe(III) and extracellular substrates such as minerals of Fe(III) and Mn(IV), particulate DMSO and electrode materials [8–12]. CymA may also provide electrons to the periplasmic octahaem tetrathionate reductase for which genomic analysis fails to find evidence for a dedicated QH2 dehydrogenase.

CymA is not in itself protonmotive. However, it may contribute to pmf generation as part of a redox loop since QH2 oxidation releases two electrons and two protons into the Q-pool via FdhABC and the electrons are vented via CymA, the periplasm. When formate feeds electrons to the Q-pool, pmf generation would appear to be solely at the level of CymA-dependent reduction of MnO2 and fumarate respectively [13]. The origin of these

Table 1 | Summary of terminal reductase systems that can operate in S. oneidensis MR-1

<table>
<thead>
<tr>
<th>Terminal electron acceptor</th>
<th>Terminal reductase</th>
<th>Site of terminal electron acceptor reduction</th>
<th>Quinol dehydrogenase</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2</td>
<td>Cytochrome c oxidase (SO4606-SO4609)</td>
<td>Inner membrane</td>
<td>Cytochrome bc1 complex (SO0608-SO0610)</td>
<td>[9]</td>
</tr>
<tr>
<td></td>
<td>Cytochrome cbb3 (SO2361-SO2364)</td>
<td>Inner membrane</td>
<td>Cytochrome bc1 complex</td>
<td>[9]</td>
</tr>
<tr>
<td></td>
<td>Cytochrome bd (SO3285-SO3286)</td>
<td>Inner membrane</td>
<td>Intrinsic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Otr (SO4144)</td>
<td>Periplasm</td>
<td>CymA</td>
<td>[23]</td>
</tr>
<tr>
<td>Fumarate</td>
<td>Fcc3 (SO0970)</td>
<td>Periplasm</td>
<td>CymA</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>IFc3 (SO1421)</td>
<td>Periplasm</td>
<td>CymA</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>FrdABC (SO0396-SO0399)</td>
<td>Periplasm</td>
<td>Intrinsic (FrdC)</td>
<td></td>
</tr>
<tr>
<td>Trimethylamine N-oxide</td>
<td>TorA (SO1232)</td>
<td>Periplasm</td>
<td>TorC (SO1233)</td>
<td>[24,25]</td>
</tr>
<tr>
<td>DMSO</td>
<td>DMSO reductase, dmsAB-1 (SO1427-SO1430)</td>
<td>Extracellular</td>
<td>CymA</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td>DMSO reductase, dmsAB-2 (SO4357-SO4360)</td>
<td>Extracellular</td>
<td>CymA</td>
<td>[26]</td>
</tr>
<tr>
<td>Insoluble Fe(III)</td>
<td>MtrCAB, Dmca, MtrDEF (SO1776-SO1782)</td>
<td>Extracellular</td>
<td>CymA</td>
<td>[27]</td>
</tr>
<tr>
<td>Soluble Fe(III)</td>
<td>????</td>
<td>Periplasm</td>
<td>CymA</td>
<td>[20,28,29]</td>
</tr>
<tr>
<td>S2O42−</td>
<td>Otr (SO4144)</td>
<td>Periplasm</td>
<td>CymA</td>
<td>[23]</td>
</tr>
<tr>
<td>SO42−, S2O42−</td>
<td>Octahaem sulfite reductase, SirA (SO0479)</td>
<td>Periplasm</td>
<td>SirCD (SO0483, SO0484)</td>
<td>[31]</td>
</tr>
</tbody>
</table>

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Figure 1 | Catalytic reduction of O₂ by CymA adsorbed on a graphite electrode

Cyclic voltammograms of a CymA film on a neomycin-coated pyrolytic graphite edge electrode in anaerobic buffer (thick line) and with buffer containing 6 μM O₂ (thin line). The reduction of oxygen directly at the electrode without CymA (broken line) is shown for comparison. Scan rate is 20 mV/s, electrode rotation is 3080 rev./min in 20 mM Mops (pH 7) containing 2 mM neomycin, 20°C. SHE, standard hydrogen electrode.

observations is unclear, but there is the possibility that the location of the terminal reduction and its specific proton-uptake stoichiometry contributes to the pmf generated when electrons exit the Q-pool via CymA.

*S. oneidensis* MR-1 exploits its respiratory flexibility to survive in redox-stratified environments where conditions fluctuate in space and time. It is likely that both genetic and metabolic mechanisms determine the path(s) of electron flux at any moment. The levels of various terminal reductases and CymA are dependent on growth conditions, illustrating genetic control [14–17]. Less is known about the mechanisms of metabolic control that may regulate the distribution of electrons to terminal reductases. One contribution may be the redox poise of the Q-pool, i.e. its effective electrochemical potential as defined by the ratio of Q to QH₂ [18]. *S. oneidensis* MR-1 contains UQs (ubiquinones) with *E°‘* ≈ +80 mV, in addition to MMQs (methylmenaquinones) and MQs (menaquinones) with *E°‘* ≈ −80 mV. In fully aerobic cells, approximately 90% of the Q-pool is UQ, but this falls to approximately 50% in anaerobic cultures (Table 2). As a consequence, lower electrochemical potentials may be imposed by the Q-pool during anaerobic growth in order that catalysis by a subset of QH₂ dehydrogenases having lower operating potentials is favoured.

### Substrate specificity and catalytic bias of CymA

Biochemical and voltammetric analysis of purified CymA have shown that it uses MQ-7, but not UQ-10, as a cofactor [6]. This is consistent with genetic experiments where deletion of the genes for MQ synthesis produced a phenotype identical with that of a cymA deletion strain [9,19]. In contrast, UQH₂ (ubiquinol) supplies electrons from the Q-pool when trimethylamine N-oxide is the terminal electron acceptor [19]. TorC, a homologue of CymA, serves as the QH₂ dehydrogenase in this case (Table 1). The QH₂-specificity of TorC is distinct from that of CymA. The origin of this specificity within the enzyme structures and its physiological relevance remain to be established. The extent to which electrons distribute freely across the distinct constituents of the Q-pool will define the extent to which the QH₂-specificity of these, and other, enzymes result in groups of enzymes driven by the electrochemical potential defined by only one of the MQ/MQH₂ and UQ/UQH₂ couples with little cross-talk.

When the catalytic performance of CymA was resolved by protein film voltammetry, a strong bias for Q reduction over QH₂ oxidation was revealed [6]. In fact, there was no discernable evidence for QH₂ oxidation despite applying a driving force greater than 0.5 V for QH₂ oxidation and that is likely to exceed that supplied by the terminal electron acceptor *in vivo*. This is consistent with predictions of catalytic bias based on the reduction potentials of the

### Table 2 | Quinone content of *S. oneidensis* MR-1 under different growth conditions

<table>
<thead>
<tr>
<th>Conditions</th>
<th>MQ-7 (%)</th>
<th>MMQ-7 (%)</th>
<th>Total UQ (%)</th>
<th>UQ-6 (%)</th>
<th>UQ-7 (%)</th>
<th>UQ-8 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fully aerobic: air flushed to mid-exponential phase at 23–25°C [19]</td>
<td>13</td>
<td>0</td>
<td>87</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microaerobic: 6 h on agar at 30°C [32]</td>
<td>41</td>
<td>6</td>
<td>54</td>
<td>2</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>Microaerobic: 15 h on marine medium, with shaking at 120 rev./min at 25°C [33]</td>
<td>44</td>
<td>6</td>
<td>46</td>
<td>1</td>
<td>11</td>
<td>34</td>
</tr>
</tbody>
</table>
CymA haems and MQ/MQH₂ couple [5]. Nevertheless, it is surprising given that CymA functions as a QH₂ dehydrogenase in vivo and in assays of the purified enzyme that measure electron transfer to a terminal reductase such as the periplasmic fumarate reductase Fcc₃.

It may be that the rate of QH₂ oxidation in the protein film voltammetry experiment is too low to measure, but sufficient to support the physiological role. However, we are exploring the possibility that salt-bridge formation and/or solvent exclusion within the electron-transfer complex formed between CymA and a terminal reductase result in altered reduction potentials and/or reorganization energies that facilitate rapid interprotein electron transfer and QH₂ oxidation. Little is known about the nature of the complexes formed between CymA and its reduct partners. Transient complexes have been implied by the diagrams that are frequently presented to illustrate the role of CymA as a hub for electron distribution to multiple terminal reductases within S. oneidensis MR-1 (e.g. [10,20,21]). There is experimental evidence to support rapidly exchanging complexes [21]. However, genetic and biochemical evidence has also been presented that argues for long-lived electron-transfer modules formed between molecules of CymA and terminal reductases [5,22]. For this scenario, the cartoon of anaerobic respiration would be better drawn with a CymA molecule for each terminal reductase. This is an area of research that warrants more detailed investigation.

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