STUDIES ON THE CYTOCHROMES BD-TYPE OXYGEN REDUCTASE SUPERFAMILY AND THE DISCOVERY OF A NOVEL NITRIC OXIDE REDUCTASE

BY

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DISSERTATION

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Respiration is the process by which an organism utilizes the reducing equivalents (electrons) produced during catabolism to create an electrochemical potential across the cellular membrane, which can then be expended to produce ATP. Living organisms, bacteria and archaea in particular have discovered numerous ways to respire using highly conserved enzyme superfamilies whose members can couple the free energy released during redox reactions to the creation of proton motive force. Combined with biochemical studies, the evolution of these superfamilies deduced from bioinformatics is very informative about the structural and functional features that are most useful to conserve energy by the creation of proton motive force. This work investigates two important enzyme superfamilies whose members are responsible for virtually all aerobic respiration on the planet—the bd-type oxygen reductase superfamily and the heme-copper oxidoreductase superfamily.

The bd-type oxygen reductases are present in bacteria and archaea and catalyze the 4-electron reduction of oxygen to water. Electrons from membrane bound quinols first reduce a low spin heme $b$ from which they are transferred to a di-heme active site where where oxygen is reduced to water. Energy is conserved by the transfer of protons from the cytoplasm to the active site which is located near the periplasmic surface. *E. coli* has two bd-type quinol oxidases, called cytochrome bd-I and cytochrome bd-II. It had previously been shown that cytochrome bd-I generates a proton motive force, but work from another group claimed that this was not the case for bd-II. As part of this work, it was demonstrated that bd-II functions identically to bd-I and generates a membrane voltage.

In a second project dealing with the cytochrome bd superfamily, genomic sequence analysis of cytochrome bd revealed a subfamily of distinct enzymes present in archaea. One of these archaeal
cytochrome \textit{bds}, was heterologously expressed in \textit{Escherichia coli}, purified and characterized. This enzyme was shown to have three hemes \textit{b} instead of two hemes \textit{b} and one heme \textit{d}, as is typical of enzymes of this superfamily. The enzyme is highly active, using ubiquinol as substrate. This is the first example in which a member of the cytochrome \textit{bd} superfamily lacking heme \textit{d} has been isolated and characterized. A third project involving the importance of a glutamic acid residue in the active site of \textit{Escherichia coli} cytochrome \textit{bd} has also been discussed.

The heme-copper oxidoreductase (HCO) superfamily contains many proton pumping oxygen reductases as well as several subfamilies of nitric oxide reductases (NORs) which convert nitric oxide to nitrous oxide. These NORs perform different chemistry than the HCOs but have a very similar protein structure and a similar active site. Significantly, the active site of purified NORs contain a Fe atom instead of the copper in the oxygen redutases. Using bioinformatics methods, a distinct new clade of NORs was identified that was previously not recognized. These enzymes have been assumed reduce nitric oxide and were thus named eNORs. One member of this clade is encoded in the genome of \textit{Rhodothermus marinus}, a thermophilic bacteroidete.

\textit{Rhodothermus marinus} was shown to possess a nitric oxide reductase by measuring the accumulation of nitrous oxide in the containers in which the bacteria is grown on nitrate. Then the enzyme was purified and characterized and confirmed to be a nitric oxide reductase. This confirms for the first time a large group of enzymes, previously not identified, that participate in denitrification in many prokaryotes.
ACKNOWLEDGEMENT

This is the end of a very special journey. Working towards the Doctor of Philosophy degree has meant more to me than just another degree. I have lived in another country to earn it, adapted to another culture and found so much of myself that I never knew before. To have a passion for learning is great but, being a scientist gives you an opportunity to constantly live that passion, to sculpt it into skills, an ability to think in a useful way and to make something tangible using it. I would never have had the opportunity to learn all of that without the opportunity accorded to me by the University of Illinois, and the inspiration and help I received from my friends and the community in Champaign-Urbana.

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# TABLE OF CONTENTS

Chapter 1: Introduction .............................................................................................................. 1

1.1 Cytochrome bd-type oxygen reductase superfamily ..................................................... 3

1.2 Heme-copper oxygen reductase superfamily of enzymes ......................................... 7

Chapter 2: Aerobic respiratory chain of Escherichia coli is not allowed to work in fully uncoupled mode .................................................................................................................. 13

2.1 Introduction .................................................................................................................... 14

2.2 Results .......................................................................................................................... 17

2.3 Discussion ....................................................................................................................... 21

2.4 Materials and Methods ................................................................................................. 25

Chapter 3: Denitrification in Rhodothermus marinus and a novel nitric oxide reductase ................................................................................................................................. 29

3.1 Introduction .................................................................................................................... 29

3.2 Results .......................................................................................................................... 31

3.3 Discussion ....................................................................................................................... 40

3.4 Materials and Methods ................................................................................................. 41

3.5 Supplementary Information ............................................................................................ 45

Chapter 4. Investigation of the role of E445 in the active site of cytochrome bd from Escherichia coli ............................................................................................................................ 47

4.1 Introduction .................................................................................................................... 47

4.2 Results .......................................................................................................................... 50

4.3 Discussion ....................................................................................................................... 57
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>Materials and Methods</td>
<td>58</td>
</tr>
<tr>
<td>4.5</td>
<td>Supplementary Information</td>
<td>62</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>63</td>
</tr>
<tr>
<td>5.2</td>
<td>Results</td>
<td>66</td>
</tr>
<tr>
<td>5.3</td>
<td>Discussion</td>
<td>72</td>
</tr>
<tr>
<td>5.4</td>
<td>Materials and Methods</td>
<td>73</td>
</tr>
<tr>
<td>5.5</td>
<td>Supplementary information</td>
<td>77</td>
</tr>
<tr>
<td>Chapter 6: References</td>
<td>82</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

Respiration is the process by which an organism uses reducing equivalents (electrons) produced during catabolism to create an electrochemical potential across its cellular membrane, which can then be expended to produce ATP or used for solute/ion exchange or ATP-driven pumps. Aerobic respiration refers to the use of oxygen as the terminal electron acceptor in the process of energy conservation. The ability to respire oxygen is very advantageous as the free energy available in the reduction of oxygen is high. (Figure 1.1)

**Standard Reduction Potential, E_m at 25 C, pH 7**

<table>
<thead>
<tr>
<th>Redox Couple</th>
<th>E_m</th>
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<tbody>
<tr>
<td>O_2/H_2O</td>
<td>0.815 V</td>
</tr>
<tr>
<td>Fe^{3+}/Fe^{2+}</td>
<td>0.77 V</td>
</tr>
<tr>
<td>NO_3^-/N_2</td>
<td>0.75 V</td>
</tr>
<tr>
<td>MnO_2/MnO_2</td>
<td>0.52 V</td>
</tr>
<tr>
<td>CO_2/CH_4</td>
<td>0.24 V</td>
</tr>
<tr>
<td>SO_4^{2-}/HS^-</td>
<td>0.22 V</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>cytc(ox/red)</td>
<td>0.25 V</td>
</tr>
<tr>
<td>UQ/UQH_2</td>
<td>0.113 V</td>
</tr>
<tr>
<td>MK/MKH_2</td>
<td>-0.075 V</td>
</tr>
<tr>
<td>O_3/O_2^-</td>
<td>-0.16 V</td>
</tr>
<tr>
<td>S^0/HS^-</td>
<td>-0.27 V</td>
</tr>
<tr>
<td>H_2O/H_2</td>
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</tr>
</tbody>
</table>

Figure 1.1 An electron tower adapted from data available in (1). The high reduction potential of oxygen makes aerobic respiration energetically favorable.
However, molecular oxygen exists in triplet state, i.e. it contains two unpaired electrons of parallel spin in its outer orbital. This means that it can only be reduced by one electron at a time. This results in the formation of reduced oxygen species which are extremely reactive and have an even higher reduction potential than oxygen itself. These reactive oxygen species (ROS) have the ability to damage biomolecules and cofactors in enzymes. (2) It is therefore essential that oxygen be completely reduced to water without the release of intermediate, toxic reduced oxygen species. Living organisms have therefore developed three highly specialized enzyme superfamilies that are capable of catalyzing oxygen reduction to water – heme-copper oxygen reductases (HCO), \textit{bd}-type oxygen reductases and alternative oxidases (AOX).

All three of these enzymes have the ability to reduce oxygen to water without releasing ROS but, they differ in their ability to harness the energy available in oxygen reduction to generate proton-motive force. (3-5) Some of the Heme-copper oxygen reductases are responsible for the most ATP generation/ O$_2$ reduced, while the \textit{bd}-type enzymes generate less electrochemical potential and alternative oxidases do not contribute to the generation of proton-motive force at all. (6-9) Within the physiological context, each of these enzymes play diverse roles in respiration, oxygen detoxification, redox control, prevention of formation of ROS etc.

With regards to aerobic respiration, this thesis deals only with the mechanism of oxygen reduction, energetic efficiency, distribution and role of \textit{bd}-type oxygen reductases. The role of heme-copper oxygen reductase family members in denitrification is discussed later.
1.1 Cytochrome bd-type oxygen reductase superfamily

Cytochrome bd-type oxygen reductase

Cytochrome bd-type oxygen reductase is a respiratory enzyme that reduces oxygen to water using electrons extracted from membrane-bound quinols. Cytochrome bd is a transmembrane protein consisting of mostly two or sometimes three subunits. (Figure 1.2) (10-12) The enzyme extracts electrons from quinol at a quinol binding site using a low spin heme b, named heme b_{558} based on its absorption maxima. (13, 14) These electrons are then transferred to a di-heme active site consisting of heme b_{595} and heme d where oxygen is reduced to water. (Figure 1.2) (15-19) The d-type heme is a chlorin which is a reduced form of heme b, a protoporphyrin. (20) (Figure 1.3)

Figure 1.2 Crystal structure of cytochrome bd-type oxygen reductase from Geobacillus thermodenitrificans. (unpublished data; personal communication from Schara Safarian)
Both the quinol binding site and the oxygen reduction site are close to the periplasm. It is believed that the protons from quinol reduction are released to the periplasm while the protons for the formation of water are obtained from the cytoplasm, using a conserved proton channel. This vectorial transfer of charges across the membrane is said to be responsible for the observed generation of membrane potential by cytochrome $bd$. (21, 22) Much of the mechanism of cytochrome $bd$ was deduced using indirect biochemical and biophysical assays and our ability to further elucidate the catalytic mechanism was severely limited by the lack of a crystal structure or similar structural information. Fortunately, our collaborators at Dr. Hartmut Michel’s lab in the Max Planck Institute at Frankfurt have recently solved the crystal structure of cytochrome $bd$ to an average resolution of $\sim 3.5\text{Å}$ (unpublished data) and this information has informed some of my later experiments.
Distribution and Role of cytochrome bd-type oxygen reductases

Cytochrome bd-type oxygen reductases are found only in bacteria and archaea. They are widely conserved in proteobacteria, firmicutes, cyanobacteria, actinobacteria and bacteroidetes, (23) Heme-copper oxygen reductases conserve more energy than bd-type oxygen reductase (6, 24) and since they are both encoded in the genome of many organisms, it begs the question – why would the organism prefer to use bd if it does not conserve as much energy? It appears that the preferential use of one oxygen reductase over the other is most often regulated based on the oxygen concentration in the environment. (25) Cytochrome bd and the C-family heme-copper oxidase have high oxygen affinity and low energetic efficiency while the A and B family heme-copper oxygen reductases have lower affinity for oxygen and translocate more protons per oxygen turned over. Nevertheless, bd oxidases play a pivotal role in some bacterial and archaeal electron transport chains, particularly where bd is the only respiratory oxygen reductase. (26-31) They have also been associated with heme biosynthesis by contributing to the generation of proton motive force and protein folding by re-oxidation of DsbB. (32, 33)

Surprisingly, cytochrome bd is present in the genomes of many ‘strict’ anaerobes. Its remarkably high affinity (0.3-0.5 µM) for oxygen (34-36), it was hypothesized, allows these organisms to adapt to microaerobic environments by scavenging oxygen using bd. (29, 37-40) This is achieved by regulating the expression of bd by Fnr, an oxygen sensitive regulator and ArcAB, which is sensitive to the redox state of quinones in the membrane and is activated under microaerobic conditions. Fnr represses the expression of the oxidases in the absence of oxygen. (25, 37, 41)
The oxygen scavenging ability of \( bd \) is particularly useful in diazotrophs for protecting enzymes sensitive to oxygen exposure like the Molybdenum containing nitrogenase. In *Klebsiella pneumoniae* and *Azotobacter vinelandii*, cytochrome \( bd \) was shown to be essential for nitrogen fixing. In *Azorhizobium caulinodans*, both \( bd \) and the C-family heme-copper oxygen reductase are both shown to be important for oxygen scavenging. (42-44)

\( Bd \) has also been judged to be part of the oxidative stress response in bacteria because of its ability to use up oxygen faster than it can bind to flavins and iron-sulfur cluster proteins, which would create ROS. In a recent paper, Imlay et al. suggest that cytochrome \( bd \) is maintained in anaerobic bacteria particularly to draw electrons and oxygen away from fumarate reductase, a prominent culprit in the production of ROS by low-potential redox centers in anaerobic bacteria. (2, 45) Furthermore, cytochrome \( bd \) was demonstrated to have low peroxidase and significant catalase activity. (46, 47) The catalase activity of \( bd \) could detoxify \( \text{H}_2\text{O}_2 \). This might explain the peroxide sensitivity of *E.coli* and *Azotobacter vinelandii* \( bd \) mutants and the enhanced resistance to peroxide in mycobacterium when \( bd \) is overexpressed. (48, 49)

\( Bd \) is found in many pathogenic and commensal bacteria including Group B *Streptococcus*, *Bacteroides*, *Chlamydia* and gram-negative proteobacteria, (26, 29, 30) It was shown to be essential during the process of colonization of the host environment as well as for the survival of some bacteria during its dormant phase in the host. (35, 50, 51) Interestingly, lactic acid bacteria (LAB) and some other bacteria, which do not produce heme use \( bd \) in a respiratory chain that becomes active in the presence of exogenous heme and quinone. (26, 27, 30, 52) It appears from the above evidence, that \( bd \) is particularly important for pathogens. Additionally, it is not homologous to any human protein and for these reasons, \( bd \) has been suggested as a possible drug target for treating bacterial infections. (23, 53)
In addition to oxidative stress, \textit{bd} has been associated with response to phosphate starvation and acidic environments and other stress conditions. (54, 55) In \textit{E. coli} and many other organisms, multiple copies of \textit{bd} exist and are regulated under different conditions. It was suggested that the different copies of \textit{bd} differed in their ability to generate proton motive force. (54) This theory has been addressed and disproved as part of my dissertation research. (56)

Most of the above evidence about the function of \textit{bd}-type oxygen reductases is relevant in bacteria. However, genes encoding \textit{bd}-type oxygen reductases are widely distributed in archaea and this thesis attempts to begin an investigation into the characteristics of a \textit{bd}-type oxygen reductase from archaea.

### 1.2 Heme-copper oxygen reductase superfamily of enzymes

**Heme-copper oxygen reductases**

Heme-copper oxygen reductases are multi-subunit transmembrane proteins, which are named for the heme and \textit{Cu\textsubscript{B}} that form a binuclear active site at which oxygen is reduced to water. The electrons for oxygen reduction are obtained from membrane bound quinols or cytochrome \textit{c}; the enzymes are accordingly called the quinol oxidase or cytochrome \textit{c} oxidase respectively. The electrons from the electron donor pass from a conserved binding site, through a low spin heme to the binuclear site where oxygen reduction happens. (57-59) (Figure 1.4) The active site includes a unique cross-linked Histidine-Tyrosine co-factor formed by a ligand to the \textit{Cu\textsubscript{B}} and a nearby Tyrosine residue. This novel co-factor is important for the rapid turnover of oxygen to water, which prevents the formation of ROS. (60)
The oxygen reductases in the HCO superfamily are grouped into three subfamilies – A, B and C. All of the enzymes in the subfamilies share a similar subunit I which conserves the binuclear active site. This is expected as the B- and C-type oxygen reductases are believed to have evolved from the A-type oxygen reductase. (58, 59) The heme-copper oxygen reductases have the unique ability to pump protons from the cytoplasm to periplasm; this is achieved using conserved proton channels. The A-type oxygen reductase has two conserved proton channels named the D and K channel, named for an important residue in each channel respectively. The reduction of one
molecule of dioxygen requires four protons and the enzyme pumps four more. The pumped protons and two of the protons required for chemistry are translocated using the D channel whereas the K channel moves two more protons required for oxygen reduction. (61, 62) (Figure 1.5)

**Figure 1.5 Proton translocation pathways in A-type heme copper oxygen reductases**

The B- and C-type oxygen reductases are missing the D channel entirely and both pumped protons and protons for chemistry are translocated using a modified K channel. While the pumping efficiency of the B family is lower than that of the A family (n= 0.5 H⁺/e⁻), the number of protons pumped by the C-type oxygen reductase is half that of the A- family cytochrome oxidase. (n= 0.5 H⁺/e⁻) (6, 24, 63, 64)

The subfamilies of oxygen reductases are distinguished by their Kₘ(O₂). The A-type oxidase has an affinity in 0.2-1 μM range whereas the B- and C-type oxygen reductases are higher with C-
family oxygen reductases having a $K_m$ as low as 4-7 nM. (65-67) The A family oxygen reductases have been found in all domains of life and are energetically the most efficient while the B-family enzymes have been found in bacteria, and archaea and have been associated with low-oxygen environments. (6, 59) Analogous to the $bd$-type oxygen reductase, the high affinity C-family enzymes are important in pathogens and also contribute to respiratory protection of nitrogenases in *Bradyrhizobium japonicum.* (66, 68)

**Heme-copper oxygen reductases and nitric oxide reductases**

The HCO superfamily consists of enzymes that perform both nitric oxide reductase chemistry as well as oxygen reductase chemistry. The nitric oxide reductases also possess a similar subunit I to the oxygen reductases. (69, 70) The binuclear active site where nitric oxide reduction occurs, however, has a heme and an iron atom, Fe$_B$ in its active site as opposed to Cu$_B$. (70-72) (Figure 1.6) Like the oxygen reductases, different NORs (nitric oxide reductases) are also capable of taking electrons from quinols and cytochromes c and transferring them via a low-spin heme to the binuclear active site. (73) Three types of NORs have thus been identified and characterized; they are the cNOR, qNOR and q-Cu$_A$-NOR. The first of these takes electrons from cytochrome c, the qNOR from quinols and the Cu$_A$-NOR was supposed to have taken electrons from both, although the presence of the Cu$_A$ site would suggest that the natural electron donor for this NOR would be a cytochrome c. (74, 75)
Figure 1.6 The active site of *P. aeruginosa* shows that the binuclear active site contains Fe instead of Cu. The absence of the Histidine-Tyrosine crosslinked cofactor is also evident. This image was made using VMD and 3hb3.pdb and 3wfb.pdb.

The cNOR and qNOR bear strong structural similarity to the C-family of oxygen reductases while the CuA-NOR is more like the B-family oxygen reductase, suggesting that the cNOR and qNOR have evolved from the C-family and the CuA-NOR from the B-family enzyme. (59) (Figure 1.7) Rather like the CuA-NOR, which was discovered in *Bacillus azotoformans*, several new families of CuA-containing NORs have been found in genomic sequences. Sequence information suggests that the active sites of these new families are quite different from the previously characterized NORs. (74, 75) In this thesis, we have identified and characterized one of these new CuA-containing NORs from *Rhodothermus marinus*. Our evidence strongly suggests that nitric oxide reduction evolved from oxygen reduction not just once (as in the evolution of cNORs and qNORs from cbb3) but twice when the CuA-NORs evolved from the B-type family.
Figure 1.7 Heme-copper oxygen reductase family. This includes the previously characterized 
Cu₄NOR from *Bacillus azotoformans* which is annotated here as bNOR.
Chapter 2: Aerobic respiratory chain of Escherichia coli is not allowed to work in fully uncoupled mode

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*Escherichia coli* is known to couple aerobic respiratory catabolism to ATP synthesis by virtue of the primary generators of the proton motive force—NADH dehydrogenase I, cytochrome bo\textsubscript{3}, and cytochrome bd-I. An *E. coli* mutant deficient in NADH dehydrogenase I, bo\textsubscript{3} and bd-I can, nevertheless, grow aerobically on nonfermentable substrates, although its sole terminal oxidase cytochrome bd-II has been reported to be nonelectrogenic. In the current work, the ability of cytochrome bd-II to generate a proton motive force is reexamined. Absorption and fluorescence spectroscopy and oxygen pulse methods show that in the steady-state, cytochrome bd-II does generate a proton motive force with a H\textsuperscript{+}/e\textsuperscript{−} ratio of 0.94 ± 0.18. This proton motive force is sufficient to drive ATP synthesis and transport of nutrients. Microsecond time-resolved, single-turnover electrometry shows that the molecular mechanism of generating the proton motive force is identical to that in cytochrome bd-I. The ability to induce cytochrome bd-II biosynthesis allows *E. coli* to remain energetically competent under a variety of environmental conditions.
2.1 Introduction

Aerobic bacteria produce ATP via (i) oxygen-independent, substrate-level phosphorylation in glycolysis and the Krebs cycle, or (ii) oxidative phosphorylation, in which the electron flow, through the aerobic respiratory electron transport chain, generates a proton motive force ($\Delta p^{\text{in}}$) which, in turn, drives the ATP synthase. The proton motive force ($\Delta p^{\text{out-in}}$, where it is specified that the gradient is measured “outside-minus-inside”) is equivalent to the transmembrane electrochemical proton gradient and has chemical ($\Delta p^{\text{out-in}}$) and electrical ($\Delta \Psi^{\text{out-in}}$) components. At 298 K, in mV units,

$$\Delta p^{\text{out-in}} = \Delta \Psi^{\text{out-in}} - 59 \Delta p^{\text{out-in}}$$

For bacteria, such as *Escherichia coli*, grown at neutral pH, the major component of the proton motive force is $\Delta \Psi^{\text{out-in}}$, which is positive outside and typically has a value in the range of 100 to 180 mV. Bacteria usually possess branched respiratory chains in which the different components are induced to allow the cells to adapt to various environmental conditions (76). One of the parameters that depends on the composition of the respiratory chain is the “bioenergetic efficiency,” which can be defined as number of charges driven across the membrane per electron used to reduce oxygen to water, ($q/e^-$). In the *E. coli* respiratory chain, the net charge transfer across the membrane is equal to the number of protons released into the periplasm ($H^+/e^-$), which can be measured.

The aerobic respiratory chain of *E. coli* (Figure 2.1) contains three enzymes that are known to generate a proton motive force: (i) the proton-pumping NADH:quinone oxidoreductase NADH dehydrogenase I (NDH-I) ($H^+/e^- =2$) (77); (ii) cytochrome bo$_3$ ($H^+/e^- = 2$) (78), the proton-pumping oxygen reductase that is a member of the superfamily of heme-copper oxidoreductases (8, 79); and
(iii) cytochrome bd-I, which is unable to pump protons but generates a proton motive force by transmembrane charge separation resulting from utilizing protons and electrons originating from opposite sides of the membrane to generate water \((H^+/e^- = 1)\)\(^{(8, 21, 22, 80, 81)}\). In addition, there are a number of substrate dehydrogenases that are not electrogenic, including the nonelectrogenic NADH:quinone oxidoreductase, NDH-II \((H^+/e^- = 0)\).

Figure 2.1 Scheme of the components of the E. coli aerobic respiratory chain, starting with NADH as the substrate. Enzyme bioenergetic efficiency is indicated as the number of protons released into the periplasm per electron \((H^+/e^-)\) ratio. NDH-I and NDH-II are the coupled and uncoupled NADH: quinone oxidoreductases, respectively, and UQ-8 is ubiquinone-8. NDH-I and NDH-II transfer electrons to UQ-8 to yield reduced UQ-8. Three quinol:oxygen oxidoreductases, cytochromes bo\(_3\), bd-I, bd-II, oxidize reduced UQ-8 and reduce O\(_2\) to 2 H\(_2\)O.

Under growth conditions with high aeration, cytochrome bo\(_3\) predominates, whereas, under microaerophilic conditions, cytochrome bd-I is the predominant respiratory oxygen reductase that is present. Conditions of carbon and phosphate starvation result in the induction of a third
respiratory oxygen reductase, cytochrome \textit{bd}-II encoded by \text{appBC} genes (also called cyxAB or cbdAB (82-84). The ability of cytochrome \textit{bd}-II to generate a proton motive force is addressed in the current work.

Whereas cytochrome \textit{bo}$_3$ and cytochrome \textit{bd}-I have been extensively studied, very little is known about cytochrome \textit{bd}-II. Cytochrome \textit{bd}-I is a two-subunit enzyme carrying three hemes: \textit{b}$\text{558}$, \textit{b}$\text{595}$, and \textit{d} (85-87). The latter two hemes are suggested to compose a unique di-heme site for the capture of \textit{O}$_2$ and its reduction to \textit{H}$_2$\textit{O}. Heme \textit{d} plays the major role in \textit{O}$_2$ binding and formation of oxygenated intermediates (17-19, 22, 88-92). In contrast, cytochrome \textit{bd}-II remains poorly studied, but it has been shown that its spectral properties closely resemble those of cytochrome \textit{bd}-I (93). The amino acid sequences of both subunits of cytochrome \textit{bd}-I and \textit{bd}-II are also highly homologous (about 60% identity).

It has been reported recently that cytochrome \textit{bd}-II does not contribute to the generation of the proton motive force (\textit{H}+/\textit{e}$^-$ = 0) (54). This was deduced from the growth properties that was genetically constructed to lack NDH-I, cytochrome \textit{bo}$_3$ and cytochrome \textit{bd}-I. The aerobic respiratory chain from NADH is constrained to NDH-II $\rightarrow$ \textit{bd}-II. By examination of the growth of this strain under glucose-limited conditions in continuous culture, it was concluded that cytochrome \textit{bd}-II does not contribute to the proton motive force (i.e., the aerobic respiratory chain is fully uncoupled). Shepherd et al. (55) have proposed that under such conditions \textit{E. coli} creates the proton motive force by means of consumption of intracellular protons during the synthesis of \textit{γ}-aminobutyric acid (GABA), which is coupled to the electrogenic uptake of anionic glutamate by the glutamate/GABA antiporter.

In this work we directly determined the ability of cytochrome \textit{bd}-II to generate a proton motive force by examining intact cells, spheroplasts, and membrane vesicles as well as purified
cytochrome \textit{bd}-II. The data definitively show that the catalytic turnover of cytochrome \textit{bd}-II does generate a proton motive force by a mechanism identical to that of cytochrome \textit{bd}-I. A previous proposal (94) that the \textit{bd}-type oxygen reductase from \textit{Azotobacter vinlandii} is not coupled has also been demonstrated not to be correct (7). It is very likely, therefore, that all members of the family of \textit{bd}-type oxygen reductases generate a proton motive force even as they may have additional roles such as scavenging \textit{O}_2 or maintaining redox balance in the cytoplasm. The data presented has import in examining the bioenergetics of other organisms which use cytochrome \textit{bd}.

\subsection{2.2 Results}

Steady-State Generation of $\Delta \Psi$ and $\Delta \text{pH}$. $\Delta \Psi$ and $\Delta \text{pH}$ measurements were performed with inverted membrane vesicles of \textit{E. coli} strain MB37 lacking NDH-I, cytochrome \textit{bo}_3, and cytochrome \textit{bd}-I. Thus, all three of the known electrogenic components of the aerobic respiratory chain are absent. NADH oxidation can proceed via the NDH-II $\rightarrow$ \textit{bd}-II branch (Fig. 2.1). Fig. 2.2A shows that the addition of ATP (trace 1) or the substrate of \textit{bd}-II [Q$_1$ plus a reductant DTT (trace 2)], results in a spectral shift of oxonol VI, indicating the formation of $\Delta \Psi$ in the inverted vesicles (inside positive). The $\Delta \Psi$ is entirely eliminated upon the addition of gramicidin, which creates channels in the membrane. These data show that $\Delta \Psi$ across the bacterial membrane is generated due to either turnover of the F$_1$F$_0$ ATPase (trace 1) or by quinol oxidase activity (trace 2). Because the only quinol oxidase present is cytochrome \textit{bd}-II, these data imply that catalytic turnover of cytochrome \textit{bd}-II generates a transmembrane potential ($\Delta \Psi$) under steady-state conditions.

Fig. 2.2B shows the responses of the dye acridine orange (AO) under the same conditions, reporting the internal acidification in the inverted vesicles, and, therefore, formation of $\Delta \text{pH}$. The addition of either ATP (trace 1) or Q$_1$ plus DTT (trace 2) results in quenching of the AO
fluorescence, demonstrating acidification of the vesicle interior. These pH changes are abolished by gramicidin. The data clearly show that catalytic turnover of cytochrome bd-II is accompanied by the release of protons on the periplasmic side of the membrane, equivalent to the inside of the inverted vesicles.

Figure 2.2 Steady-state generation of Δp by the inverted membrane vesicles from an *E. coli* strain engineered to contain cytochrome *bd*-II as the only terminal oxidase. (A) ΔΨ generation measured with 25 mM HEPES + BIS-TRIS propane at pH 7.5, 1 mM MgCl₂, 2 µM oxonol VI, and vesicles (72 µg protein/ mL). The arrows show that the reaction was initiated by addition of 1 mM ATP (trace 1) or 50 µM Q₁ in the presence of 1 mM DTT and
Figure 2.2 (contd.) 6.25 mM (NH₄)₂SO₄ (trace 2). The ΔΨ was then dissipated by the addition of 1 µg/mL gramicidin (Gr). (B) Acidification of the vesicle interior measured with 100 mM HEPES KOH at pH 7.5, 50 mM K₂SO₄, 5 µM AO, vesicles (36 µg protein/mL), and 1 µM valinomycin. The reaction was initiated by addition of 0.1 mM ATP in the presence of 10 mM MgSO₄ (trace 1) or 1 mM DTT in the presence of 50 µM Q₁ and 1 mM MgSO₄ (trace 2). The generated ΔpH was then dissipated by 1 µg/mL gramicidin (Gr), as indicated by the arrows.

It is noted that the observed kinetics of the generation of ΔΨ and ΔpH are different (Fig. 2.2). This is because ΔΨ is produced almost immediately, whereas ΔpH develops at a much slower rate since the membrane vesicles are loaded with concentrated buffer.

H⁺/e⁻ Ratio Measurement. Figure 2.3 shows the change in the pH of an anaerobic suspension of spheroplasts from E. coli MB37 (lacking NDH-I, cytochrome bo₃ and cytochrome bd-I) upon addition of aliquots of O₂. By calibrating with HCl, the number of released protons can be calculated as 0.94 ± 0.18 H⁺/e⁻. The data is an average of nine measurements with two different spheroplasts preparations. As shown in Fig. 2.3A, under identical conditions virtually the same ratio (1.0 ± 0.07 H⁺/e⁻) can be obtained with spheroplasts from strain MB30, in which NDH-I, cytochrome bo₃, and cytochrome bd-II have all been removed. The only possible source of released protons in strain MB30 is cytochrome bd-I. This data is an average of 12 measurements with two different spheroplast preparations. The H⁺/e⁻ stoichiometry for cytochrome bd-I is in agreement with that reported earlier (8). O₂ pulses were also performed with whole cells at pH 7.3 and the results are the same as with the spheroplasts at pH 5.0. The proton release from spheroplasts, by either cytochrome bd-I or cytochrome bd-II, is inhibited by the addition of aurachin (C1-10), an inhibitor of the quinol oxidases.
Figure 2.3 Proton release from spheroplasts prepared from (A) *E. coli* strain MB30 [ΔcyoB ΔappB ΔnuoB] in which cytochrome *bd*-I is the only terminal oxidase present; and (B) *E. coli* strain MB37 [ΔcyoB ΔcydB ΔnuoB] in which cytochrome *bd*-II is the only terminal oxidase present. For conditions, see the text. O₂ was added as air-saturated water (25 °C, 1 atm).
**Single-Turnover Generation of Membrane Potential.** Figure 2.4 shows the time course of single-turnover $\Delta \Psi$ generation by the liposome incorporated, fully reduced cytochrome $bd$-II during its reaction with $O_2$. The electrometric kinetics consists of an initial lag-phase reflecting a nonelectrogenic process, followed by an electrogenic phase. Fitting the kinetics with a sequential-step reaction model (95) gives two electrically silent steps with time constants ($\tau$) of 2.1 and 7.3 $\mu$s, respectively, for the lag phase, and two steps with $\tau$ of 70 $\mu$s and 440 $\mu$s and with their relative amplitudes of approximately 90% and 10%, respectively, for the electrogenic phase. Similar kinetics of $\Delta \Psi$ generation by cytochrome $bd$-I has been demonstrated previously under the same conditions (80). In the latter work, based also on the concomitant time-resolved optical measurements, the two nonelectrogenic steps have been correlated to the transition from the reduced heme d (state R) to the heme d oxy-complex (state A) followed by the formation of the heme d peroxy complex (state P). The two electrogenic steps correlate with the sequential conversion of the peroxy intermediate to the ferryl species (state F) and then to the oxidized species (state O). Note that the $F \rightarrow O$ transition occurs only in a small fraction of the enzyme that contains bound quinol. For this reason, this reaction is associated with a relatively small electrogenic amplitude (10% of the total voltage). The data demonstrate that the two enzymes, $bd$-I and $bd$-II, function in the same manner.

### 2.3 Discussion

The current work was inspired by the report by Bekker et al. (54) concerning the function of cytochrome $bd$-II in the E. coli aerobic respiratory chain. By constructing a strain (MB37; cyo, cyd, nuo) in which the only terminal oxidase is cytochrome $bd$-II, aerobic respiration utilizing this enzyme was clearly demonstrated and quantified during continuous culture under glucose-limited growth conditions. If all three terminal oxygen reductases were absent, no oxygen utilization was
Figure 2.4. Single-turnover generation of the transmembrane electric potential during the reaction of the fully reduced, reconstituted cytochrome *bd-II* from *E. coli* with O$_2$. Conditions: 100 mM MOPS-KOH at pH 7.2, 10 µM hexaammineruthenium, 5 µM N,N,N',N'-tetramethyl-p-phenylenediamine, 50 mM glucose, 0.5 mg/mL catalase, 1.5 mg/mL glucose oxidase and 1% CO. The reaction was started by a laser flash (time τ=0, indicated by a dashed line) 400 ms following the injection of 50 µL of oxygen-saturated buffer (½O$_2$ ¼ 1.2 mM) at 21 ºC. The fit line is a best fit result for a consecutive-step reaction model with two lag-phases (electrically silent steps, τ, 2.1 µs and 7.3 µs, respectively) and two ΔΨ generation steps (τ, 70 µs, and contribution, 90%, and τ, 440 µs, and contribution, 10%, respectively). The respective phases are indicated by arrows. The time constants for *bd-I* are taken from ref. 10.
observed and the strain (MB44) grows by homolactate fermentation (54). Despite the fact that the strain in which cytochrome \textit{bd}-II is the only terminal oxidase consumes oxygen, a detailed analysis resulted in the conclusion that cytochrome \textit{bd}-II does not contribute to the proton motive force (54). Hence, according to these data, strain MB37 lacks all three of the respiratory components that contribute to the proton motive force, leaving open the question of how ATP is generated. Substrate-level phosphorylation was suggested (54) and, subsequently, it was suggested that a proton motive force could be generated by the operation of an electrogenic antiporter that imports anionic glutamate and exports newly synthesized, neutral GABA (55). According to this interpretation, \textit{E. coli} cells have the capacity to insert into its respiratory chain a terminal oxidase that is maximally coupled to the generation of the proton motive force (\textit{bo}_3: \text{H}^+/e^- = 2), minimally coupled (\textit{bd}-II: \text{H}^+/e^- = 0), or intermediate (\textit{bd}-I: \text{H}^+/e^- = 1), depending on the physiological need.

In the current work, the ability of cytochrome \textit{bd}-II to generate a proton motive force was directly measured. Contrary to the expectation based on the continuous culture experiments (54), it is definitively shown that catalytic turnover of cytochrome \textit{bd}-II does generate a proton motive force. Steady-state ubiquinol-1 oxidase (\text{H}^+/e^- = 1). The same result was obtained with cytochrome \textit{bd}-I (Figure 2.3A) but is half the value reported previously for cytochrome \textit{bo}_3 (8), which is a proton pump. Hence, these data indicate that cytochrome \textit{bd}-II, like cytochrome \textit{bd}-I, is not a proton pump. The scheme in Figure. 2.5 shows how this stoichiometry is attained. Charge separation accompanies catalytic turnover of the enzyme because the electrons and protons used to reduce \text{O}_2 to 2 \text{H}_2\text{O} come from opposite sides of the membrane. Quinol is oxidized at a site near the periplasmic surface, releasing protons into the periplasm. The protons used to form \text{H}_2\text{O} are taken from the cytoplasm delivered across the membrane by a putative proton conducting channel.
Figure 2.5. Schematic model of coupling between respiration and phosphorylation in *E. coli* in which the aerobic respiratory chain in which cytochrome *bd*-II is the only site that can generate a proton motive force. Shown are cytochrome *bd*-II (green) and the F$_1$F$_0$-ATP synthase (pink) embedded in the cytoplasmic membrane (yellow). The same diagram applies also to cytochrome *bd*-I. The proton motive force is composed of two components that depend on the magnitude of the transmembrane electric potential difference (ΔΨ) and the pH gradient across the membrane. In *vivo*, the pH gradient is determined by the buffering of the external environment and by pH homeostasis mechanisms to control the internal pH. In inverted membrane vesicles, the small internal volume is readily acidified by proton release from the oxidation of ubiquinol. The transmembrane potential is postulated to be formed by the vectorial uptake of protons from the cytoplasmic side of the membrane to the di-heme (*b$_{593}$-d) active site to form water. The resulting proton motive force is used by the F$_1$F$_0$-ATP synthase to produce ATP from ADP and inorganic phosphate.
Single-turnover electrometric experiments with the purified cytochrome \( bd \)-II incorporated in proteoliposomes allowed us to elucidate which of the partial reactions in the catalytic cycle are coupled to the generation of membrane potential. The \( \Delta \Psi \) transient caused by the reaction of the fully reduced cytochrome \( bd \)-II with \( O_2 \) consists of an initial, nonelectrogenic part (the lag-phase) followed by the electrogenic part (Figure 2.4). The lag phase is the sum of the two faster, nonelectrogenic processes and the electrogenic part consists of two slower electrogenic transitions with the first phase contributing the most to the \( \Delta \Psi \). The phases and rate constants are similar to those activity by inverted vesicles of strain MB37, in which cytochrome \( bd \)-II is the only quinol oxidase, is coupled to the generation of both the \( \Delta \Psi \) and \( \Delta p\text{H} \) component of proton motive force (Figure 2.2). Because inverted vesicles from this strain also contain a well-coupled \( F_0F_1 \)-ATPase, both \( \Delta \Psi \) (Figure 2.2A, trace 1) and \( \Delta p\text{H} \) (Fig. 2.2B, trace 1) can also be generated by addition of ATP. Hence, in the MB37 cells, aerobic respiration must be coupled to ATP synthesis via the cytochrome \( bd \)-II terminal oxidase.

### 2.4 Materials and Methods

**Cell Culture.** The *E. coli* strains MB37 (BW25113 \( \Delta \text{cyoB} \Delta \text{cydB} \Delta \text{nuoB}, \) kanamycin marker removed) and MB30 (BW25113 \( \Delta \text{cyoB} \Delta \text{appB} \Delta \text{nuoB}, \) kanamycin marker removed) containing cytochrome \( bd \)-II and cytochrome \( bd \)-I as the sole terminal oxidase, respectively (54), were a kind gift of M. Bekker (University of Amsterdam). The cells were grown aerobically in a 25 L fermentor with stirring (at aeration rate of 20 L/min) or in flasks on a shaker (at 200 rpm) in LB medium at 37 °C for 4–5 hrs until the late exponential phase.

**Enzyme Isolation.** Cytochrome \( bd \)-II was purified following a protocol described for cytochrome \( bd \)-I (32), with modifications. The MB37 membranes were solubilized with sucrose monolaurate
After solubilization, the membranes were centrifuged (160,000 g × 60 min, 4 °C), the pellet was discarded and the supernatant loaded on a DEAE-Sepharose Fast Flow column equilibrated with 50 mM potassium phosphate buffer also containing 25 mM KCl, 5 mM EDTA, and 0.1% sucrose monolaurate, pH 6.5. The elution was performed with a KCl gradient (25–470 mM). The fractions with an absorbance ratio of $A_{412}/A_{280} \geq 0.7$ were collected and concentrated.

**Steady-State Generation of ΔΨ and ΔpH.** For these measurements, inverted membrane vesicles were prepared. The cells were converted into spheroplasts by treatment with 0.1 mg/mL lysozyme in 200 mM Tris-HCl, 2 mM EDTA at pH 8.0, and 30% sucrose. The spheroplasts were pelleted at 5,000 g × 10 min, suspended in 100 mM HEPES-KOH, 50 mM K$_2$SO$_4$, 10 mM MgSO$_4$ at pH 7.5, 2 mM dithiotreitol (DTT), and 0.5 mM phenylmethanesulfonyl fluoride and sonicated. The cell debris was removed by centrifugation (at 5,000 × g for 10 min). The supernatant was ultracentrifuged at 120,000 × g for 30 min. The membrane vesicles were suspended in 25 mM HEPES + BIS-TRIS propane at pH 7.5 and used the same day. Steady-state generation of ΔΨ by the vesicles was monitored by the spectral shift of the lipophilic anionic dye oxonol VI. The absorption changes were followed in dual wavelength kinetics mode (588–625 nm) using a Shimadzu UV-3000 spectrophotometer. Acidification of the interior of the vesicles was followed by quenching of fluorescence of the pH-sensitive membrane-permeable dye AO. Measurements were carried out at 21 °C using a Hitachi F-4000 fluorescence spectrophotometer using 493 nm (excitation) and 530 nm (emission). The response was expressed as the percentage of the fluorescence intensity before addition of the substrate.

**Measurement of H$^+$/e$^-$ Ratio.** The measurement was performed both with spheroplasts and intact cells. Spheroplasts were prepared using lysozyme and EDTA as in ref. (96), collected by
centrifugation and then resuspended in 0.5 mM HEPES-KOH at pH 5.0, 100 mM KSCN, and 150 mM KCl. Then spheroplasts were pelleted once again and resuspended in the same medium. Proton release by whole cells was studied at pH 7.3, whereas experiments with spheroplasts were performed at the pH value of 5.0 to optimize their stability (97). The results were similar whether intact cells or spheroplasts were examined. Spheroplasts were used to determine if the proton translocation and oxidase activities were inhibited by aurachin (C1-10), a potent inhibitor of cytochrome bd-I (98, 99) that does not readily penetrate the outer membrane of intact cells. Proton release by spheroplasts was measured as follows. The spheroplast suspension (100 µL) was diluted into 2.5 mL of 0.5 mM HEPES-KOH at pH 5.0, 100 mM KSCN, and 150 mM KCl. Dissolved oxygen was removed by passing a stream of water-saturated argon over the solution in a sealed chamber (at 25 °C) equipped with a glass pH-electrode (Thermo Russell pH electrode, type CMAW711). Traces of oxygen were removed from the solution by letting the spheroplasts respire with endogenous substrates. Endogenous substrates are also responsible for the respiration during the oxygen pulse used to measure the proton release. To measure the H⁺/e⁻ ratio, 10 µL of air-saturated water (250 µM) was injected into the solution and the pH change was recorded. After reequilibration, 10 µL of a 1 mM HCl solution was injected into the sample and the pH change was recorded as a calibration of the system. The number of protons ejected from the spheroplasts upon the reduction of O₂ contained in 10 µL of airsaturated water (250 pmoles/µL) was calculated by noting the amount of HCl required to elicit the same change in pH. The addition of aurachin (C1-10) to a final concentration of 80 µM eliminated proton translocation for both cytochrome bd-I and cytochrome bd-II in the spheroplast preparations.

**Oxidase Activity Assay.** Oxidase activity was measured by monitoring the depletion of dissolved oxygen as a function of time using a Clark-type electrode (YSI Inc.) at 37 °C after the addition
of 100 µL of a suspension of either whole cells or spheroplasts to 1.7 mL of the same buffer used for proton-pumping measurements.

**Single-Turnover Generation of ΔΨ.** Reconstitution of cytochrome *bd*-II into liposomes, preparation of the anaerobic samples, and the direct electrometric measurements with a microsecond time resolution were performed essentially as reported (refs. 8–11 and references therein).

**Heme and Protein Concentration.** The heme *d* content was measured from the reduced-minus-“air-oxidized” difference absorption spectra using Δε_{628-607} of 10.8 mM⁻¹ cm⁻¹ (20). Protein concentration was determined by the BCA Protein Assay Reagent kit (Pierce) with bovine serum albumin as a standard.

**Data Analysis.** MATLAB (The Mathworks) and Origin (OriginLab Corporation) were used for data manipulation and presentation.
Chapter 3: Denitrification in *Rhodothermus marinus* and a novel nitric oxide reductase

3.1 Introduction

Nitrous oxide is an important greenhouse gas, comparable to CO$_2$ and CH$_4$ in the damage it causes. Its effect is particularly concerning because it is very stable in the atmosphere, with a lifetime of a 114 years. The Ozone Depletion Potential (ODP) for N$_2$O is similar to that of chlorofluorocarbons and N$_2$O emission is now the major cause of ozone depletion (100, 101). Most of the N$_2$O released into the atmosphere comes from anthropogenic activities like agriculture. In agriculture, nitrogen containing compounds are used as fertilizers and microbial processes like denitrification and nitrification produce N$_2$O. It has recently also been shown that a significant amount of nitrous oxide is released by nitrifying and denitrifying bacteria in the oceans, particularly in the oxygen minimum zones (102). In this context, studying the microbial generation of N$_2$O is imperative. In this paper we present evidence for the discovery of a new enzyme responsible for the production of N$_2$O from NO, as part of the denitrification pathway in *Rhodothermus marinus*.

Denitrification is the process of reduction of nitrate to nitrogen by a series of enzymes present in bacterial and archaeal electron transport chains. (Figure 3.1) Sometimes this pathway is terminated at the conversion of NO to N$_2$O, which is not further reduced to N$_2$. This is referred to as incomplete denitrification. Nitrous oxide reductase which reduces N$_2$O to N$_2$ is inhibited by oxygen and therefore it is typical for this final reduction step to be absent under higher concentrations of oxygen (102, 103). In this chapter, we describe the denitrification of an aerobic denitrification pathway in *Rhodothermus marinus*. 
Figure 3.1 Schematic of denitrification pathway. The nitrate reductase can sometimes also be a transmembrane protein.

As discussed in Chapter 1, the Heme-copper oxygen reductase superfamily contains many enzymes which perform either oxygen reductase or nitric oxide reductase chemistry. This enzyme superfamily has been shown to have many divergent members, which have modified versions of the active site, proton channels and electron donor site which allow the enzymes to adapt to novel environments while performing either the oxygen or nitric oxide reduction chemistry \((6, 59)\). There are three families of oxygen reductases – A, B and C, where B and C families evolved from A. Of the three NOR families previously studies, cNOR and qNOR appear to have evolved from the C family, while the Cu\(\alpha\)NOR evolved from the B-family oxygen reductase. Using Genomics and structural modeling, several new families performing nitric oxide reduction were hypothesized, all of them supposed to have evolved from the B family oxygen reductase \((59)\). (Figure 3.2) In this work, we have verified that one of the hypothesized family of NORs does in fact perform nitric oxide reduction chemistry.
Figure 3.2 Heme-copper oxygen reductase superfamily. New families eNOR, bNOR, sNOR and gNOR are represented.

3.2 Results

Denitrification in *Rhodothermus marinus*:

*Rhodothermus marinus* is a bacteroidete from the family *Rhodotherrmaceae*. It was isolated from an Icelandic hot spring, and is a thermohalophile (104). While the water from hot springs does not contain nitrate, it does contain ammonia and, ammonia oxidizers have been found in hot springs to convert ammonia to nitrate. This nitrate can then be reduced to nitrous oxide or nitrogen (105). A few thermophilic denitrifiers have been thus far identified (106, 107). In the original cultivation studies, nitrate reduction was not observed, however some genes for denitrification are present in *Rhodothermus marinus* DSM 4252. (Figure 3.3) The genes for the periplasmic nitrate
reductase NapAB are encoded for in the genome and so are the genes for the copper-dependent nitrite reductase (nirK) and the cytochrome cd₁-containing nitrite reductase (nirS). The genes known for nitric oxide reduction (cNOR and qNOR) are conspicuously absent. However, the genes for reduction of nitrous oxide, the product of the reaction that would be catalyzed by the NORs is present. We believe that the absence of genes encoding for previously characterized NORs is because the function is performed by an uncharacterized enzyme, the eNOR that was hypothesized by Hemp et al. (59).

![Denitrification diagram](image)

**Figure 3.3 KEGG pathway in nitrogen metabolism in *Rhodothermus marinus* DSM 4252.**

Created with use of Data analysis software on the IMG site.

Here, we show that *Rhodothermus marinus* reduces NO₃⁻ to N₂ under aerobic conditions. When grown under denitrifying conditions (in the presence of 30 – 50 mM NO₃⁻), NO₃⁻ utilization is observed (data not shown). Further, the accumulation of nitrous oxide is observed when organisms grown under denitrifying conditions are inoculated incubated for 30 minutes. (Figure 3.4) This suggests very strongly that nitrous oxide is formed as an intermediate during denitrification, suggesting that there exists a nitric oxide reductase. When the above incubation is performed with acetylene, excess nitrous oxide is accumulated, indicating the presence of nitrous oxide reductase which is inhibited by acetylene. Our data not only confirms that *Rhodothermus marinus* is a denitrifier but, suggests that a novel nitric oxide reductase (not homologous to previously characterized NORs) is present in the organism.
Figure 3.4 Nitrous oxide production by *Rhodothermus marinus* during denitrification. *R. marinus* grown under denitrifying conditions, was inoculated into fresh medium incubated in septum stoppered bottles and incubated for 30 minutes at 65 °C. The headspace was then analyzed for nitrous oxide accumulation in the presence and absence of acetylene, an inhibitor of nitrous oxide reductase.

**Biochemical characteristics of eNOR:**

A nitric oxide reductase of the designated eNOR family was expressed in *Rhodothermus marinus* by growing it under denitrifying conditions, and then purified in its native form. A very closely related eNOR was purified earlier from *Magnetospirillum magnetotacticum* (originally *Aquaspirillum magnetotacticum*) but was not tested for nitric oxide reductase activity. The eNOR from *Rhodothermus marinus* in a partially pure state (Figure 3.5) was shown to have two subunits – subunit I of approximately 40 kDa and subunit II of around 20 kDa. The theoretical prediction
of molecular weight for subunit I is 62 kDa but, it is not unusual for membrane proteins to run at a lower weight than expected. Moreover, the electrophoresis pattern is similar to the one observed for the protein purified from *Magnetospirillum* (108). The identity of the *Rhodothermus marinus* eNOR was ascertained by LC/MS analysis after peptide digestion. In the peptide analysis, 24 % of subunit I’s sequence was covered with a MASCOT score of 354 and 60 % of subunit II’s sequence was covered with a MASCOT score of 2022. (Details of MS analysis in Supplementary information)

![Figure 3.5 Electrophoretic analysis of eNOR via SDS-PAGE gel.](image)

The eNOR was then shown to have nitric oxide reductase activity with a turnover number of 0.68 ± 0.21 NO/s (n= 4) and virtually no oxygen reductase activity (less than 0.07 O₂/s). (Figure 3.6) The NO reductase activity was confirmed by establishing N₂O production as a result of eNOR enzymatic activity, by using Gas chromatography followed by Electron Capture Detection (ECD).
Detection by ECD is an established method for detecting nitrogen oxides in a gaseous mixture. (Figure 3.7)

**Figure 3.6** Nitric oxide reduction activity of eNOR using PMS and Ascorbate as electron donors. Analyzed used WPI ISO-NOP electrodes.

**Figure 3.7** Nitrous oxide is the product of the reaction between eNOR and NO in the presence of PMS and Ascorbate.
The heme co-factors in eNOR:

Since the eNOR is similar to the B-family oxidase, it was expected to have two heme co-factors like the $ba_3$ oxidase-eNOR. While the heme-copper oxidase family enzymes were previously found to use $a$, $a_6$, $b$ or $o$ hemes, the eNOR is suspected to have unusual co-factors (59, 109). The UV-visible spectra of the heme co-factors in this eNOR as well as the proposed NOR from *Magnetospirillum* are unique. (Figure 3.8a and (108)) The pyridine hemochrome spectra largely indicate the presence of only one type of heme, which when extracted by pyridine absorbs at 583 nm, as in the earlier work. The $\alpha$-band of the hemes is close to, however, blue-shifted in comparison to other $a$-type hemes. (Figure 3.8b)

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**Figure 3.8a** The UV-visible spectral properties reveal a novel heme-cofactor $b$. The pyridine hemochrome assays reveals a unique absorption peak.

When analyzed by LC/MS, the elution profile of extracted hemes from eNOR reflects a different pattern from those of heme $a$ from bovine heart and heme $b$. (Figure 3.9) The molecular weight of the eNOR heme was found to be around 647 Da. Theoretically, the structure that would most approximate this weight could use two formyl modifications in the place of methyl groups in a $b$-type heme. (This would add up to a molecular weight of 644 Da.) However, this observation
of a novel heme will require further and more rigorous analysis before it can be confirmed. The question of which metal occupies the binuclear active site has not been resolved in this work. However, previous observations about the heme-copper oxidase superfamily suggest that copper is present in the active site when the histidine tyrosine crosslink is present and iron is present when other ligands are present. The sequence and structural alignment of the eNOR suggest that there is no tyrosine, which that could act as the crosslink.

Figure 3.9 Elution profile of hemes extracted from a) bovine cytochrome c oxidase b) cytochrome ba3 from Thermus thermophilus and c) eNOR.
Structural characteristics of eNOR using a structural model

A computationally generated model of the eNOR on the i-TASSER server was found to align very well with the crystal structure of \( ba_3 \) oxidase from \( \textit{Thermus thermophilus} \) (PDB code: 1XME). The RMSD of the structure was found to be within 2 Å, and a number of structural features were clearly seen to be conserved. The ligands to the Cu\(_{B}\) (H241, H290, and H291) and the heme (H77, H398) in the binuclear site correlate to the corresponding geometric positions in \( ba_3 \) though, the side chains are not directly superimposable. The numbering used in the figure is based on the amino acid numbering in the eNOR. It is evident that no Tyrosine exists in the model that could serve as a crosslink to the Histidine ligand to the Cu\(_{B}\), as in the oxygen reductases. Instead, a Glutamine (Q245) takes its place and we suggest that it could act as a ligand to the Fe\(_{B}\), like the Glutamate in the cNOR and qNOR \( (70, 72, 110) \). The ligand (H396) to the second heme in subunit I is identical to Histidine 384 in \( ba_3 \). Ligands to the Cu\(_{A}\) site in subunit II (H126, C161, C165, H169 and M172) are also present.

Most interestingly, there are three completely conserved residues \( (Y256, S318Y280 \text{ and H}321E330) \) which are very similarly placed to residues in the K channel of the \( ba_3 \) oxidase \( (Y248, S309 \text{ and T}312 \text{ respectively}) \) \( (24) \). S269 is in a similar position to T315 and is 98 % conserved. \( (\text{Figure 3.10, 3.11}) \) (Sequence analysis data in supplementary information) There is a Glutamate in an equivalent position in subunit II to E15 from \( ba_3 \). Y280 is completely conserved and is in the vicinity. There is no geometrically equivalent residue in the \( ba_3 \) oxidase. We think that it is quite possible that these residues constitute a proton channel for the transfer of protons from the cytoplasm to the active site. If that is true, it is possible that the eNOR could be electrogenic, which has been previously suggested by Hemp \textit{et al.} \( (59) \). This has also been suggested of the qNOR but, has not yet been proven. \( (72, 73) \)
Several other residues near the active site are conserved – S318, H321, Y232, N384 and H388 – are also completely conserved, but, it is not immediately apparent what their functions are.

Figure 3.10 Subunits I of *T. thermophilus ba₃* and *R. marinus* eNOR indicating the presence of a K-channel for translocation of protons from the cytoplasm to periplasm.

Figure 3.11 The conserved proton channel in a) *ba₃* and b) in the putative proton pathway in eNOR.
3.3 Discussion

Genomics has long predicted the existence of a number of uncharacterized families of heme-copper oxygen reductases (58). Structural modeling and previous biochemical evidence have led to the prediction of function for several of these new families as nitric oxide reductases (59). Prior to these predictions, a novel Cu_A containing NOR was purified and characterized from *Bacillus azotoformans* (75, 111). This NOR is closer in structure to the B-family oxygen reductase than the C-family oxygen reductase and is expected to have evolved from the B-family oxygen reductase. According to the notation presented here, this would be the bNOR. Most of the proposed new families of oxygen reductases are likewise expected to have evolved from the B-family oxygen reductase. We have in this work, verified the existence of one of these family of nitric oxide reductases, the eNOR. This has interesting implications for the study of denitrifiers since this NOR was isolated from an obligate aerobe (112). Denitrification typically occurs under anaerobic conditions or in oxygen minimum zones (102). Further more, the possibly formylation of the cofactor in eNOR is also interesting as the enzyme responsible for formylation of hemes is only produced under aerobic conditions(113). The use of denitrification in aerobic conditions and their impact on the niches, denitrifiers possessing the eNOR can occupy requires further consideration.

Our structural model and sequence analysis of eNOR reveals conserved polar residues which could form a possible proton channel. The bNOR from *Bacillus azotoformans* has been shown to have a conserved proton channel, which contributes to proton motive force generation (114). Likewise, we suggest that eNOR could be electrogenic. This would have serious implications for questions of energy conservation in denitrification as this would significantly increase the amount of energy that can be conserved by denitrification.
3.4 Materials and Methods

Growth and Expression Conditions

*Rhodothermus marinus* DSM 4252 was inoculated from frozen stock and grown in 5 ml of DSM Medium 630 with 10 g/L NaCl at 60 °C for 36 hrs. It was then inoculated into a larger secondary culture and grown overnight. 25 ml of the culture was inoculated into 1 L of medium with 30 mM nitrate added. The cells were shaken at 75 rpm and grown at 60 °C. The cells were collected by centrifugation at 8000 rpm.

Purification of eNOR

The culture of *Rhodothermus marinus*, once harvested, was resuspended in 100 mM Tris-HCl, pH 8 with 10 mM MgCl₂ and 50 µg/ml DNase, using a Bamix homogenizer. The resulting solution was spun down at 42000 rpm in a Beckman Ultracentrifuge. The membrane pellet was collected and resuspended in 20 mM Tris-HCl, pH 7.5, 1 % CHAPS (Affymetrix) to a final concentration of 40-50 mg/ml. The solution was stirred at 4 °C for 1 hr. In this step a lot of peripheral membrane proteins appear to be solubilized and the remaining protein is pelleted by spinning down at 42000 rpm for 1 hr. The remaining pellet is then solubilized in 20 mM Tris-HCl, pH 7.5, 1 % DDM (Affymetrix) at a final protein concentration of around 5-10 mg/ml. The DDM solubilized fraction was once again centrifuged at 42000 rpm to pellet down protein that was not solubilized.

The solubilized protein was then loaded on a DEAE CL-6B (Sigma) column, pre-equilibrated in 20 mM Tris-HCl, pH 7.5, 0.05 % DDM, and subjected to a linear gradient spanning from 0 to 500 mM NaCl. The fraction containing the NOR, which eluted at around 200 mM salt, was then loaded on a Q Sepharose High Performance (GE Healthcare) column, pre-equilibrated
with 20 mM Tris-HCl, pH 7.5, 0.05 % DDM and then eluted in a gradient from 0 to 1 M NaCl. The eNOR was eluted at around 250 mM salt and the eluted fraction was then loaded on a Chelating Sepharose (GE Healthcare) column, loaded with Cu$^{2+}$ and equilibrated with 20 mM Tris-HCl, 500 mM NaCl, as previously described for cytochrome $ca{a}_3$ from $Rhodothermus marinus$ (115). The NOR, identified by a spectroscopic peak at 591 nm was once again loaded on a Q Sepharose High performance column, and a gradient was run between 0 and 300 mM NaCl and the first peak was found to be the eNOR.

**Gel Electrophoresis**

The purified eNOR was run on a Tris-Hepes 4-20 % acrylamide gel (NuSep) in the recommended Tris-Hepes-SDS running buffer at 120 V for ~1 hr. The protein was viewed with respect to the Precision Plus Protein™ Dual Color Standards (BIO-RAD).

**UV-Visible Spectroscopy**

All spectra were recorded on a HP Agilent 8453 UV-Vis spectrophotometer using a quartz cuvette from Starna Cells (No. 16.4Q-10/Z15). Potassium Ferricyanide was used to obtain the oxidized spectrum, and dithionite was used to obtain the reduced spectrum.

**Pyridine Hemochrome Assay**

The hemes in eNOR were analyzed using a pyridine hemochrome assay (116). A stock solution of 200 mM NaOH with 40 % pyridine was prepared. The stock solution was mixed 1:1 with the protein and an oxidized spectrum was obtained by adding 3 µl of 100 mM K$_3$Fe(CN)$_6$. A reduced spectrum was similarly prepared by adding a few crystals of sodium dithionite. The reduced minus oxidized spectrum was used to identify the heme co-factors.
Heme extraction and HPLC Analysis

The hemes from eNOR were extracted and analyzed using an HPLC elution profile according to established protocols (117, 118). 50 µl of eNOR was mixed with 0.45 ml of acetone / HCl (19:1) and shaken on a rotary shaker for 20 minutes at room temperature. The mixture was centrifuged at 14000 rpm for 2 minutes, followed by addition of 1 ml of ice cold water, and 0.3 ml of ethyl acetate to the supernatant. The water/ethyl acetate mixture was vortexed and centrifuged again for 2 minutes. The ethyl acetate phase was recovered and concentrated using a speed vac.

The extracted hemes were analyzed using a Hewlett-Packard 1090 Series HPLC by separation on a DeltaPak C18 column, and an acetonitrile (0.05 %TFA) / water (0.05 % TFA) gradient from 50 to 100 %.

NO reductase activity verification using GC

Anaerobic reaction conditions were set up in a 5 ml clear serum vial (Voigt Global Distribution, Inc) sealed with a 20 mm rubber stopper, by passing N₂ through 2 ml of 20 mM KPi, 0.05 % DDM, pH 7.5 with 1 mM TMPD, 5 mM Ascorbate. A control was performed by adding only 50 µM NO. Sample reactions were begun by adding eNOR to a final concentration of 100 nM. The reaction was incubated at 42 °C for half an hour before the head space was injected into an HP Agilent 5890 Series GC, fitted with a TCD and ECD (SRI Instruments) for verification of N₂O production.

Turnover measurement using a Clark electrode

A sealed chamber fitted with an ISO-NO (World Precision Instruments) electrode was used for NO reductase activity measurements. 1 mM TMPD or 100 µM PMS and 4 mM Ascorbate were was added to 2 ml 50 mM Citrate, pH 6, 0.05 % DDM in the reaction chamber and all traces of
oxygen were removed by passing water-saturated Argon for 20 minutes through the solution. The buffer system also contained an oxygen scavenging system constituting 100 nM catalase, 35 nM Glucose oxidase and 90 nM Glucose. The NO reduction traces were recorded using a Duo-18 (World Precision Instruments), and activities calculated from the slope of the traces.
Figure 3.12 Mass spectrometric analysis of eNOR subunits.
Figure 3.13: Sequence alignment for sequences from the B-family oxygen reductase and eNOR. This highlighted sequences calls attention to the conserved active site motif (HXXVY) which binds to Cu_B and (HXXQQ) which we expect would bind Fe_B.
Chapter 4. Investigation of the role of E445 in the active site of cytochrome $bd$
from *Escherichia coli*

4.1 Introduction

Cytochrome bd-type oxygen reductases have a unique active site, standing in contrast to the better understood heme-copper oxygen reductases which incorporate a heme and copper in their active site. Cytochrome bd-type oxygen reductases have three heme co-factors to catalyze the reduction of oxygen to water (11). Cytochrome $bd$ extracts electrons from a membrane-bound quinol at a quinol binding site, using heme $b_{558}$, a low spin heme. The electrons are then transferred to a di-heme active site consisting of two high spin hemes – heme $b_{595}$ and heme $d$ (17). Heme $d$ is a chlorin, a reduced protoporphyrin which is a rare co-factor, found only in cytochrome bd-type oxidases, cd1-type nitrite reductases and catalases (20).

Cytochrome $bd$ is a two (or sometimes three) subunit transmembrane protein. The third subunit, cydX, is conserved in all most all proteobacteria, but appears to be absent in other organisms (12, 119, 120). Almost all the conserved amino acid residues found to be essential for catalysis, thus far, have been found in subunit I, cydA (23, 121) while subunit II, cydB and its rapid evolution has been associated with specific adaptations of $bd$ in the presence of toxic compounds such as cyanide (122, 123). All the above co-factors have binding sites in subunit I though subunit II is found to be essential for the binding of hemes $b_{595}$ and $d$ (124). All of the insights into the catalytic mechanism of cytochrome $bd$ have been obtained using evidence from biophysical and biochemical studies, particularly using the enzyme from *Escherichia coli*. The recently obtained crystal structure of cytochrome $bd$ from *Geobacillus stearothermophilus* (unpublished data, courtesy of Schara Safarian from Max Planck Institute – Frankfurt) should help us make further strides in the elucidation of the mechanism of oxygen reduction. Another factor
that was delaying mechanistic study was the lack of a robust method for purification of cytochrome \textit{bd}. The previous methods required several steps of purification but Hoeser \textit{et al.} have recently prepared a construct that allows for purification of cytochrome \textit{bd} in a single step using a strep tag (125). In this work, we characterize \textit{E. coli} cytochrome \textit{bd} using site-directed mutagenesis and, some biochemical and biophysical methods to understand its active site structure.

Figure 4.1 Crystal structure of cytochrome \textit{bd} from \textit{Geobacillus thermodenitrificans}. \textit{E. coli} numbering is used for residues.
Active site structure

The residues binding low-spin heme $b_{558}$ were found to be His186 and Met393 using site-directed mutagenesis as well as NIR-MCD and EPR spectra (126-128). High spin heme $b_{595}$ was shown to be bound to His19 (128, 129). The ligands to heme $d$ have, however, remained in question. It was proposed that E99 could be a potential ligand to heme $d$ but the recent crystal structure clearly refutes such a hypothesis (130, 131). In fact E445 is in close proximity to the Fe center of heme $d$, and could possibly be a ligand. (Figure 4.2) This is a flimsy hypothesis unlikely, however, since a previous mutation of E445 into alanine did not disturb heme $d$ (132). Paradoxically, it damaged heme $b_{595}$ instead. Similarly, a mutation of E99, a residue shown to be near to the Fe in heme $b_{595}$ was shown to disturb heme $d$ rather than heme $b_{595}$ (130). All of this supports the idea that heme $b_{595}$ and $d$ are closely linked in their redox activity for which there is a lot of supporting evidence from spectroscopy, MCD and electrochemistry (17, 19, 133).

Role of E445 in the catalytic mechanism of cytochrome $bd$

A mutation of E445 to alanine led to the perturbation of heme $b_{595}$. Originally this was interpreted as the absence of heme $b_{595}$ in the protein. Both the EPR spectrum of the oxidized protein and the absence of the bond between His19 and heme $b_{595}$ from Resonance Raman spectra were interpreted as absence of the heme (132). However, a later study used CO-bound flash photolysis of E445A and EPR spectra of reduced protein conclude that the reduction of cytochrome $bd$ had instead been disturbed by the mutation. A convincing model was proposed which hypothesized that two residues in the vicinity of the heme $b_{595}$ and $d$ were required to be protonated upon reduction and that E445 was one of these. E99 was possibly the other. In E445A, the presence of E99 allowed for the reduction of heme $d$ but not, heme $b_{595}$ (22). The crystal
structure indicates that E99 and E445 are far apart, dissuading us from the above hypothesis. A previous Resonance Raman spectrum had also shown that three carboxylate groups were protonated upon reduction (129). E107, E99 and E445 are some of the candidate protonatable carboxylate groups corresponding to this evidence.

Figure 4.2 Binding sites of hemes d and b_{595} with E445 and E99 in close proximity, respectively.

### 4.2 Results

**UV-visible spectra of E445 mutants**

Four mutations in the residue E445 were made using a Strep-tag construct previously prepared (125) – E445A, E445C, E445H and E445Q. Air-oxidized (Figure 4.3) and dithionite reduced (Figure 4.4) spectra of each of these mutants shows a diminished 595 nm and 440 nm signal which correspond to reduced heme b_{595}. In addition, the mutant E445H also had a reduced heme d signal. There is a peak at 606 nm in the air oxidized spectra of E445A, whose origin is not yet understood. It has not been commented on before in literature. The reduced spectra also clearly
reflect a lower absorbance of the peak at 560 nm but this is also explained by the perturbation of $b_{595}$ since heme $b_{595}$ does absorb at this wavelength.

**Figure 4.3** UV-visible spectra of wild type cytochrome $bd$ and E445 mutants as prepared. The spectra were normalized to the peak at 412/413 nm and a baseline correction was applied.
The aberrant peak in the oxidized spectra of E445A is not oxygen-bound heme $b_{595}$

In the E445A mutant, the glutamate is replaced by alanine. This suggests that the oxygen has more room around the active site and could possibly diffuse past heme $d$ and bind heme $b_{595}$. In order to discount the possibility that the aberrant peak at 606 nm was oxygen-bound heme $b_{595}$,
E445A was deoxygenated and an optical spectrum was obtained. However, it was found that the aberrant peak at 606 nm does not disappear upon deoxygenation. (Figure 4.5, Figure 4.6)

**Figure 4.5** Deoxygenation of WT cytochrome *bd* shows that the peak corresponding to the oxygen-bound heme is 646 nm. Air-oxidized protein is deoxygenated by repeated freeze-thaw cycles in the presence of Argon and then incubation in an anaerobic glove box. The protein is then reduced with Titanium dioxide.
Deoxygenation of E445A shows that the peak corresponding to the oxygen-bound heme is 646 nm. Air-oxidized protein is deoxygenated by repeated freeze-thaw cycles in the presence of Argon and then incubation in an anaerobic glove box. The protein is then reduced with Titanium dioxide. The 606 nm peak does not disappear upon deoxygenation showing that it does not correspond to oxygen bound heme $b_{595}$.

**A glutamine mutation in the place of E445 recovers oxygen reduction activity**

The mutants E445A, E445C, E445H and E445Q were tested for oxygen reduction activity. Remarkably, E445Q was the only moderately active enzyme showing an oxygen reduction activity that was ~15% of WT $bd$. (Table 4.1) Previous evidence suggested that E445 played a role in
catalysis stabilizing the reduced heme $d$ by being protonated upon reduction (22). Glutamine, in 
E445Q is not protonatable, suggesting that E445 may play a slightly different role or a more 
complex one than was previously believed.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Oxygen reduction activity ($e^-/s$)</th>
<th>% WT activity</th>
<th>Oxygen reduction activity/heme $b$ ($e^-/s$)</th>
<th>% WT activity</th>
<th>%KCN inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT $bd$</td>
<td>1466 ± 173</td>
<td>100.00</td>
<td>737±108</td>
<td>100.00</td>
<td>25.6</td>
</tr>
<tr>
<td>E445A</td>
<td>12 ± 2</td>
<td>0.85</td>
<td>9±2</td>
<td>1.24</td>
<td>94.5</td>
</tr>
<tr>
<td>E445C</td>
<td>44 ± 7</td>
<td>2.98</td>
<td>33±7</td>
<td>4.52</td>
<td>25.9</td>
</tr>
<tr>
<td>E445H</td>
<td>19 ± 2</td>
<td>1.32</td>
<td>19±3</td>
<td>2.63</td>
<td>37.4</td>
</tr>
<tr>
<td>E445Q</td>
<td>218 ± 11</td>
<td>14.87</td>
<td>84±6</td>
<td>11.48</td>
<td>25.6</td>
</tr>
</tbody>
</table>

Table 4.1 Oxygen reduction activity of E445 mutants.

A histidine mutation of E445 greatly perturbs the heme $d$ binding site

A heme analysis was performed on each of the mutants using pyridine hemochrome analysis and estimating the heme $d$ content using the reduced minus oxidized spectra of each of the mutants. (Table 4.2) In E445H, heme $d$ appears to be lost.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Heme $b$ ($\mu$M)</th>
<th>Heme $d$ ($\mu$M)</th>
<th>Heme $b$/Heme $d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT $bd$</td>
<td>10.55</td>
<td>5.30</td>
<td>1.99</td>
</tr>
<tr>
<td>E445A</td>
<td>49.00</td>
<td>35.89</td>
<td>1.37</td>
</tr>
<tr>
<td>E445C</td>
<td>34.09</td>
<td>29.05</td>
<td>1.17</td>
</tr>
<tr>
<td>E445H</td>
<td>8.43</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E445Q</td>
<td>14.50</td>
<td>5.62</td>
<td>2.58</td>
</tr>
</tbody>
</table>

Table 4.2 Heme analysis of E445 mutants using pyridine hemochrome spectral analysis.
Electrochemical analysis of hemes in E445 mutants

As previously noted, the reduction potential of hemes \( d \) and \( b_{595} \) have been shown to be inter-connected (19, 133). It would then be natural to expect that the midpoint potential of heme \( d \) would be affected by the drastic changes we can see to the heme \( b_{595} \) binding site. Yet surprisingly in E445A and E445C, the midpoint potential of heme \( d \) is only mildly affected with an increase or decrease of 20 mV respectively. In the E445Q however, the midpoint potential is significantly reduced, by about 50 mV. This can be explained by the replacement of E445 by Q, a residue that cannot be protonated. Histidine and Cysteine are also likely to be protonated but it is possible that the pK\(_a\) in the context of the active site may vary.

E445Q is the only one of the mutants for which the midpoint potential of heme \( b_{595} \) can be calculated as the reduction of heme \( b_{595} \) in E445C and E445A do not appear to be optically apparent. (Table 4.3) The midpoint potential of heme \( b_{595} \) has increased to 194 mV, indicating that some changes in the heme environment have made it easier to reduce.

Surprisingly, the modification of E445 into Q has also drastically affected the midpoint potential of heme \( b_{558} \). Given the proximity of E445 to heme \( b_{558} \), it is possible that E445 affects the redox state of heme \( b_{558} \) and perhaps mediates electron transfer between heme \( b_{558} \) and heme \( d \).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Heme ( b_{558} ) (mV)</th>
<th>Heme ( d ) (mV)</th>
<th>Heme ( b_{595} ) (mV)</th>
<th>Detergent used</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT ( bd )</td>
<td>88.30</td>
<td>246.06</td>
<td>133.70</td>
<td>DDM</td>
</tr>
<tr>
<td>E445A</td>
<td>46.90</td>
<td>265.59</td>
<td></td>
<td>DDM</td>
</tr>
<tr>
<td><strong>E445Q</strong></td>
<td><strong>183.00</strong></td>
<td><strong>199.16</strong></td>
<td><strong>194.00</strong></td>
<td><strong>DDM</strong></td>
</tr>
<tr>
<td>WT ( bd )</td>
<td>183.00</td>
<td>289.00</td>
<td>134.00</td>
<td>SML</td>
</tr>
<tr>
<td>E445C</td>
<td>156.00</td>
<td>222.65</td>
<td></td>
<td>SML</td>
</tr>
</tbody>
</table>

Table 4.3 Midpoint potentials of hemes in WT cytochrome \( bd \) and E445 mutants.
4.3 Discussion

The crystal structure of cytochrome $bd$ strongly supports a number of the theories previously suggested on the basis of biochemical evidence. The ligands of hemes $b_{558}$ and $b_{595}$ have been verified and the residues that were expected to contribute to proton uptake (S140, E99, E107, H126) do appear to form a proton channel from the cytoplasm to periplasm.

Most interestingly the hemes $d$ and $b_{595}$ appear to be extremely close to one another ($<3$ Å) as the biochemical evidence had suggested. (Figure 4.7) However, heme $b_{558}$ is also within electron transfer distance of both hemes $d$ and $b_{595}$ (~8 Å). Given the close proximity of all three hemes, it is likely that the proton transfer which stabilizes electron transfer is an integral factor to the electron transfer scheme. E445 is a protonatable residue very near to heme $d$. However, it also lies directly in the middle of heme $b_{558}$ and heme $d$. (Figure 4.7)

Figure 4.7 Tri-heme active site of cytochrome $bd$ showing that the closest distance between heme $b_{595}$ and $d$ is 3.27 Å. The ring-to-ring distances between heme $b_{558}$ and heme $d$, and heme $b_{595}$ and $d$ are 10.3 Å and 6.11 Å respectively.
Our electrochemical evidence suggests strongly that the midpoint potential of heme $b_{558}$ is affected by E445. This might indicate a role for E445 in the proton coupled electron transfer between heme $b_{558}$ and heme $d$. E445 is said to be at around 3 Å from heme $d$ and 6.95 Å from a propionate group of heme $b_{558}$. If oxygen is bound to heme $d$, it is likely that E445 moves closer to heme $b_{558}$, possibly making a hydrogen bond to heme $b_{558}$. It is not clear in what way conformational changes or protonation state of E445 would affect heme $b_{558}$, but it seems that it does affect its midpoint potential. Further experiments such as looking at the difference in protonation states between WT and E445 mutants using Resonance Raman and FTIR would be required to clarify these details.

### 4.4 Materials and Methods

**Site directed mutagenesis of cytochrome bd oxidase from Escherichia coli**

The construct previously prepared for purification of WT cytochrome $bd$ with a strep tag was used as a template (125). Mutagenesis primers were designed for point mutations at E445. (primer information in supplementary Table S1) The Quikchange mutagenesis kit (Stratagene) was used for PCR amplification. The plasmid with a mutation was transformed into XL-10 Gold cells and incubated overnight at 37 °C to recover colonies. The plasmid sequence was verified by submission for sequence verification with ACGT technologies.

**Cell Growth and Protein Purification**

A single colony was inoculated into 5 ml of LB (yeast extract and tryptone were purchased from Acumedia and NaCl from Sigma-Aldrich) with 100 µg/ml Ampicillin and incubated with shaking at 37 °C. The following day, the 5 ml culture was inoculated in 300 ml LB with 100 µg/ml
Ampicillin and grown overnight at 37 °C. On the third day, 10 ml of the secondary culture was inoculated into twenty four 2 L flasks containing 1 L LB with 100µg/ml Ampicillin, each. The flasks were incubated at 37 °C while shaking at 200 rpm, until the OD600 of the culture reached 0.6. The temperature was then lowered to 30 °C, and the culture was incubated for 8 hrs or overnight.

The fully grown cultures were then pelleted by spinning down at 8000 rpm for 8 minutes, in 500 ml centrifuge bottles. The harvested cells were then resuspended in 100 mM Tris-HCl, 10 mM MgSO₄, pH 8 with DNaseI and a protease inhibitor cocktail from Sigma. The cells were then homogenized using a Bamix Homogenizer, and passed through a Microfluidizer cell at 100 psi, three times, to lyse the cells. The soluble fraction of the lysate was then separated from the insoluble by spinning down the lysate at 8000 rpm. Membranes were extracted from the soluble fraction by centrifuging the soluble fraction at 42000 rpm for 4 hours.

Membranes were resuspended in 50 mM sodium phosphate, 300 mM NaCl, pH 8 and then solubilized with 1% DDM or 1% SML. The solubilized membranes were spun down at 42000 rpm for 45 minutes to remove unsolubilized membranes. The supernatant was stirred with Strep-tactin resin (QIAGEN) for 1 hr and then loaded onto a column. The column was washed with 20 column volumes of 50 mM sodium phosphate, 300 mM NaCl, pH 8, 0.05% DDM. The protein was then eluted with 2.5 mM desthiobiotin in the above buffer. The fractions with protein were pooled in concentrated using an Amicon concentrator with a 100 kDa molecular weight filter. The concentrated protein was exchange in 50 mM sodium phosphate, 0.05% DDM, pH 7. This protein was used for further studies.
UV-visible spectroscopy

Spectra of the protein were obtained using an Agilent DW-2000 Spectrophotometer in the UV-visible region. The cuvette used has a pathlength of 1 cm. The oxidized spectrum was taken of the air-oxidized protein. The enzyme was reduced with dithionite to obtain a reduced spectrum.

Collection of Pyridine Hemochrome spectra and Heme Analysis

For the wild type or mutant enzymes, 35 ul of the enzyme solution was mixed with an equal volume of 40% pyridine with 200 mM NaOH. The oxidized spectra were measure in the presence of ferricyanide and the reduced in the presence of dithionite. The values of heme b were calculated according to the matrix suggested in (116). The concentration of heme d was estimated using the extinction coefficient $\varepsilon_{(629-670\,\text{nm})} = 25\,\text{mM}^{-1}\text{cm}^{-1}$.

Measurement of oxygen reductase activity

Oxygen reductase activity was measured using the Mitocell Miniature Respirometer MT200A (Harvard Apparatus). 5 mM DTT and 250 µM Q1 were used as electron donors to measure oxygen reduction by the E445 mutants and wild type cytochrome bd. 300 µM KCN was used to test the cyanide sensitivity of the enzymes.

Electrochemical measurement of midpoint potential of the hemes

A potentiometer was connected to an Agilent DW-2000 Spectrophotometer to measure the solution potential and optical spectra simultaneously. A Calomel electrode is connected to a transparent cuvette and the electrode is then connected to a potentiometer. The cuvette contains a 2.5 ml solution consisting of a cocktail of redox mediators, each at a final concentration of 10 µM and the protein. The solution is poised at a redox potential of 440 mV using potassium ferricyanide
and then successively reduced using a fresh sodium dithionite solution. At each successive
reduction, an optical spectrum is obtained to monitor the reduction of the hemes optically. The
data was analyzed by fitting the data to the following function using OriginPro 2016 or
Matlab R2015a.

\[ y = \frac{A_1 + A_0}{1 + 10^{0.012 (X - E_{1/2}) / 0.59}} - A_0 \]

The wavelength combinations used for the heme analysis were taken from which allowed for
resolution of the individual optical spectra of the hemes (133).
### 4.5 Supplementary Information

Table 4.4 Primers for preparation of E445 mutants

<table>
<thead>
<tr>
<th>Primer</th>
<th>Tm</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecolibd_E445A-f</td>
<td>70.8</td>
<td>GGCTGGTTGCGTGGCTGCGTATGGGCGGCAACCG</td>
</tr>
<tr>
<td>Ecolibd_E445A-r</td>
<td>70.8</td>
<td>CGGTGGCGGCGCATAAGCCACGAACCCAGCC</td>
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Chapter 5: Isolation and Characterization of a \( bb' \)-type oxygen reductase from *Caldivirga maquilingensis*

5.1 Introduction

Archaea were the last domain of life to be discovered, following eukaryotes and bacteria (134). Archaea were originally found in extreme conditions of pH, temperature and salinity. Although they have since been discovered in more temperate habitats, it is clear that archaea have been the most successful of the three domains in adapting to harsh conditions. Since their discovery, scientists have been attempting to put newly discovered knowledge about Archaea, in the context of concepts established in the other two kingdoms of life. Archaeal transcription and translation machinery are similar to those of Eukaryotes, but like bacteria they are capable of many different kinds of metabolism (135-137). They are the only kingdom capable of methanogenesis and utilize many types of anaerobic respiration as well as aerobic respiration. While a number of respiratory complexes are homologous to those found in bacteria and eukaryotes, the adaptation of these proteins to the extreme niche of archaea is very important to consider.

Archaea have most of the proteins that constitute the aerobic respiratory chain of bacteria except complex I. Succinate-quinone oxidoreductases (138), cytochrome \( bc_1 \) complexes (139) and heme-copper oxygen reductases from archaea have been characterized (140, 141). Cytochrome \( bd \)-type respiratory oxygen reductases are the other major family of terminal oxidases, besides the heme-copper oxygen reductase superfamily. The presence of cytochrome \( bd \) has been noted but no functional member of the cytochrome \( bd \) family in archaea has ever been established, according to the best of our knowledge (142-145).
Cytochrome \( bd \) is an enzyme that converts oxygen to water using three hemes, unlike the HCO enzymes which use two hemes and copper. Cytochrome \( bd \) accepts electrons from quinols using a low-spin heme \( b_{558} \) and transfers these electrons to a di-heme active site containing two high spin hemes. In some of the characterized cytochrome \( bd \) enzymes, these active site hemes were shown to be one heme \( b \) and one heme \( d \), but some other isoforms were shown to contain only hemes \( b \). Those cytochrome \( bd \) family members that contain only hemes \( b \) were named the cyanide insensitive oxidase (CIO) or cytochrome \( bb' \) oxidase, and have been identified in \textit{Pseudomonas aeruginosa}, \textit{Bacillus subtilis} and others. No CIO has ever been isolated and characterized. Cytochrome \( bd \) has been shown to have two subunits in many organisms, and three subunits in others. The function of this third subunit is still being investigated but it has been implicated in the stability of heme \( d \) and also in contributing to the activity of the enzyme. Cytochrome \( bd \) has been shown to generate proton motive force by translocation of protons from the cytoplasm to the periplasm. This has been hypothesized to occur using a conserved proton channel.

In this study we demonstrate the existence of a functional cytochrome \( bd \)-type oxidase in the archaeon, \textit{Caldivirga maquilingensis}. \textit{Caldivirga maquilingensis} is the only identified member of the genus \textit{Caldivirga}, belonging to family \textit{Thermoproteaceae}, of the order \textit{Thermoproteales}. \textit{Caldivirga maquilingensis} was isolated from a hot spring in Phillipines, and grows optimally at 85 °C and at a pH between 3.7-4.2. We show that the isolated enzyme is highly thermostable, consistent with origin in a hyperthermophile. This \( bd \) family member is also shown to be a cyanide insensitive oxidase (CIO) containing only hemes \( b \). Its unique biochemical and structural characteristics are discussed, including its novel subunit II, which is homologous to subunit I. This archaeal cytochrome \( bb' \) is widely distributed in archaea, including several organisms previously
thought to be strictly anaerobic. We believe that the metabolic adaptations of archaea and their aerobic respiratory chains need to be reconsidered in the context of existing and functional cytochrome $bb'$ oxygen reductases.

**Distribution of cytochrome $bd$-type oxygen reductases**

Cytochrome $bd$-type oxygen reductases have been found in bacteria and archaea. A genomic analysis of the cytochrome-$bd$ type oxygen reductases reveal many different phylogenetic groups of the enzyme. (Figure 5.1)

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**Figure 5.1** Cytochrome $bd$-type oxygen reductase superfamily representing the different phylogenetic groups.
Distinct families of the enzyme were revealed including a family of enzymes prominently present in the Bacillus clade, a family of enzymes in Archaea and a most unique family in sulfate reducers such as *Shewanella* which have an enzyme that appears to have the oxygen reduction site conserved but, attached to a cyt c binding site instead of a quinol binding site. (Supplementary Figure S1) The Bacilli enzymes appear to have some distinct features in the active site. The enzymes unique to archaea have a subunit II that seems to be structurally related to subunit I rather than the other subunits II. In fact, a close look at the crystal structure from *Geobacillus thermodenitrificans* (refer Chapter 4, Figure 4.1) shows some similarity between the subunits I and II of the *bd*. A further study of these factors in the evolution of this enzyme superfamily would be needed for us to better understand these observations. The purification of an enzyme from Archaea and with the crystal structure, our ability to make assessments of the importance of subunit II would help us begin that investigation.

5.2 Results

**Purification and spectroscopic characterization of the bb’ from *C. maquilingensis***

Cytochrome *bb’* from *Caldivirga maquilingensis* consisted of two genes – *cydA* and *cydB*. The protein encoded by *cydA* was homologous to subunit I of *Escherichia coli* cytochrome *bd*. As previously mentioned, the product of *cydB* is also homologous to subunit I. The operon was cloned into the pET22b vector and expressed in an *Escherichia coli* strain, CBO (C43, Δ*cydA ΔappB*) (125). *Escherichia coli* has two cytochrome *bd* –type oxygen reductases encoded in the genome; CBO has both knocked out. The enzyme from *Caldivirga* were engineered to have numerous different tags – 6xHistidine, FLAG, GST and GFP. None of the above tags were successful, either because of a poor yield of protein or because of the inability of the affinity-tagged proteins to bind to columns with their corresponding epitopes. A GFP-tagged protein was used to verify the
expression of cytochrome $bb'$-type oxygen reductase from *Caldivirga maquilingensis*. The presence of the protein could be noted by following the fluorescence of the protein under UV light. The purified protein was verified by mass spectrometry. Subunit I from *Caldivirga maquilingensis* $bb'$ was revealed in the mass spectrum. (Supplementary Figure S3)

A reduced-minus-oxidized spectrum of $bb'$ reveals the absence of the heme $d$ absorbance peak. The presence of heme $b_{595}$ is also not apparent in the spectrum; there is no maxima at 595 nm and the soret peak at 440 nm is also missing. (Figure 5.2)

![UV-visible spectra of cytochrome bd from *Escherichia coli* and bb’ from *Caldivirga maquilingensis*.](image)

**Figure 5.2 UV-visible spectra of cytochrome bd from *Escherichia coli* and bb’ from *Caldivirga maquilingensis*.**

**Pyridine hemochrome spectra of bb’**

Hemes were extracted from cytochrome $bb'$ of *Caldivirga maquilingensis* as described previously (116). It was clearly shown that the only kind of hemes in the protein were $b$-type hemes. (Figure 5.3) This was verified by analyzing the hemes in the protein by LC-MS. (data not shown)
Figure 5.3 Pyridine hemochrome spectra of *Escherichia coli* bd and *Caldivirga maquilingensis* bb’.

**Oxygen reduction activity of cytochrome bb’ and cyanide insensitivity**

The oxygen reduction activity of cytochrome bb’ was tested using a clark electrode, with reduced coenzyme Q1 (reduced using DTT) as the electron donor. (Table 5.1) At 37 °C the activity is ~330 e⁻/s (/heme b). While this is not as high as the activity of *E. coli* bd at the same temperature (over 1000 e⁻/s), the enzymatic activity is still significant, particularly considering the fact that the source of the enzyme is a thermophilic organism whose growth is optimum at 65 °C. The oxygen reductase activity of cytochrome bb’ was also cyanide insensitive, at 150-250 μM KCN, a
concentration of cyanide that would inhibit the activity of heme-copper oxygen reductases (23). This is a characteristic feature of cytochrome \textit{bd}-type oxygen reductases, and is a characteristic that does not appear to be affected by the absence of heme \textit{d}. The enzyme is also susceptible to Aurachin AC1-10, a known inhibitor of cytochrome \textit{bd} at concentrations as low as 250 nM (146). (Supplementary Figure S2)

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Sample & Oxygen reduction activity \((e^/-s)\) \\
\hline
\textit{Escherichia coli bd} & 333 ± 20 \\
\textit{Caldivirga maquilingensis bb'} & 1065 ± 73 \\
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\end{tabular}
\caption{Oxygen reduction activity of cytochrome \textit{bd} and cytochrome \textit{bb’} in the presence of 350 \(\mu\)M coenzyme Q1 and 5 mM DTT.}
\end{table}

**Structural model of cytochrome \textit{bb’} oxygen reductase from \textit{Caldivirga maquilingensis}**

A structural model of cytochrome \textit{bb’} was created using the Swiss PDB server. In accordance with the similarity and high degree of conservation between the subunits I from different organisms, the model of subunit I was easily obtained. Subunit II of cytochrome \textit{bb’} could only be modelled by using subunit I as a template, a result in agreement with the closer similarity of subunit II from \textit{bb’} with the well-conserved subunit I. A closer look at the active site of \textit{Caldivirga maquilingensis} reveals that a highly conserved W441 has been substituted by Alanine. This substitution has occurred a number of times in \textit{Thermoproteales}. Comparing the active sites of \textit{Caldivirga maquilingensis} and \textit{Geobacillus thermodenitrificans} also shows us that a few charge residues around the heme \textit{d} binding sites are missing. E371 and Y375 are replaced by I388 and V392. Sequence analysis of all the active sites in cytochrome \textit{bd}-type oxygen reductases reveal that the residues near the heme \textit{d}- binding site in a majority of the enzymes from proteobacteria
around the well-conserved GWxxxExGRxPW site are polar. In archaea and some *Bacilli*, residues are replaced by hydrophobic residues. The effect of this change in the active site may have on the function of the enzyme is not obvious and must be further investigated.

Figure 5.4 Structural model of subunits I from *Geobacillus thermodenitrificans* and *Caldivirga maquilingensis* respectively. The boxed regions reveal more polar residues in *Geobacillus*, colored by red. Green represents aromatic residues.
**Similarity between subunit I and subunit II**

A close look at subunit II of $bb'$ from *Caldivirga maquilingensis* reveals that subunit II is structurally similar to subunit I. The first four helices of subunit II, which form the oxygen reduction site, are similar to the first four helices of CydA, the canonical subunit I of cytochrome $bd$-type quinol oxidase. (Figure 5.5) The features constituting the binding site of heme $b_{558}$ and the quinol binding site are conspicuously absent. This unique form of subunit II is present only in archaea.

**cydA**

```
  TMH1  TMH2  TMH3  TMH4  TMH5  TMH6  TMH7  TMH8  TMH9
```

**cydA - homolog**

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  TMH1  TMH2  TMH3  TMH4  TMH5  TMH6  TMH7  TMH8
```

Figure 5.5 Subunit I of $bb'$ from *Caldivirga maquilingensis* is represented by a scheme of TM helices. Subunit II, is represented as cydA- homolog, a modified version of subunit I.

A phylogenetic analysis of subunits II and subunits I reveals that these subunits II cluster separately and seem to be related to a modified subunit I present in *Rubrococcus sp.* (Figure 5.6) It is not possible to make conclusions about the evolutionary history of this unique subunit II based on this phylogenetic analysis. Further understanding of the role of subunit II would be useful towards this end.
Figure 5.6 Phylogenetic tree of cydA and cydA-homologs showing that they cluster separately. cydA-homologs are more closely related to a modified subunit I present in actinobacteria.

5.3 Discussion

The presence of cyanide insensitive oxidases containing only hemes $b$ has been reported previously in literature but no active $bd$-type enzyme has ever been purified with only $b$-type hemes. In this work, we have demonstrated the purification of an active enzyme from *Caldivirga maquiltingensis* which contains only $b$-type hemes. This demonstration eliminates the possibility
that in the previous studies in membranes, the d heme signal may have been masked because of
the enzymes’ low abundance in membranes.

Observation of the in-situ conversion of b-type heme into d-type heme in Catalase II (147, 148) from Escherichia coli has led to speculation that a similar reaction is responsible for the conversion of heme b to heme d in the active site of bb’-type enzymes. Further investigation of the active site in bb’ from Caldivirga may lead to answers for that question. Our structural models of the enzyme reveal that there are fewer polar residues in the heme d binding site of Caldivirga. Whether this affects the function and properties of the enzyme would also need to be tested.

Finally, we have shown for the first time that a cytochrome bb’-type oxygen reductase encoded for in archaea is active. The presence of these enzymes in a number of archaea specializing in surviving in hypoxic environments (142) suggests that the enzyme might be pivotal in the adaptation of these archaea to hypoxic habitats.

5.4 Materials and Methods

Preparation of construct for of cytochrome bd oxidase from Escherichia coli

The genes encoding the bb’ oxygen reductase (Gene Object ID: 641276193-4) from C.
maquilengensis were PCR amplified using primers purchased from Integrated DNA Technology. The genes were cloned into pET22b (Invitrogen) using 5’ NdeI and 3’ XhoI cut sites. The inherent 6-Histidine tag in the vector was used to purify the protein. The vector was engineered to use EGFP, GST or FLAG tags alternatively. The tag was added to subunit II in case of EGFP and FLAG; a tag on both subunit I and II was attempted for the His-tag and GST tag. The expression
vector, along with pRARE (Novagen) was then transformed into (CBO ΔcydBΔappC::kan) for protein expression.

**Cell Growth and Protein Purification**

A single colony was inoculated into 5 ml of LB (yeast extract and tryptone were purchased from Acumedia and NaCl from Sigma-Aldrich) with 100 µg/ml Ampicillin and incubated with shaking at 37 °C. The following day, the 5 ml culture was inoculated in 300 ml LB with 100 µg/ml Ampicillin and grown overnight at 37 °C. On the third day, 10 ml of the secondary culture was inoculated into twenty four of 2L flasks containing 1 L LB with 100 µg/ml Ampicillin, each. The flasks were incubated at 37 °C while shaking at 200 rpm, until the OD600 of the culture reached 0.6. The temperature was then lowered to 30 °C, and the culture was incubated for 8 hrs or overnight.

The fully grown cultures were then pelleted by spinning down at 8000 rpm for 8 minutes, in 500 ml centrifuge bottles. The harvested cells were then resuspended in 100 mM Tris-HCl, 10 mM MgSO4, pH 8 with DNaseI and a protease inhibitor cocktail from Sigma. The cells were then homogenized using a Bamix Homogenizer, and passed through a Microfluidizer cell at 100 psi, three times, to lyse the cells. The soluble fraction of the lysate was then separated from the insoluble by spinning down the lysate at 8000 rpm. Membranes were extracted from the soluble fraction by centrifuging the soluble fraction at 42000 rpm for 4 hours.

Membranes were resuspended in 20 mM Tris, 300 mM NaCl, pH 8 and then solubilized with 1% DDM or 1% SML. The solubilized membranes were spun down at 42000 rpm for 45 minutes to remove unsolubilized membranes. The supernatant was stirred with Ni-NTA resin for 1 hr and then loaded onto a column. The flow through was shown to contain the bb’ because of its
poor affinity for the nickel column. The flow through was then diluted in buffer to contain 50 mM salt and then loaded onto a DEAE column equilibrated with 20 mM Tris, pH 8, 0.05% DDM. An elution gradient was run between 0-500 mM NaCl and bb' was partially purified and used for assays and spectroscopy.

**UV-visible spectroscopy**

Spectra of the protein were obtained using an Agilent DW-2000 Spectrophotometer in the UV-visible region. The cuvette used has a pathlength of 1cm. The oxidized spectrum was taken of the air-oxidized protein. The enzyme was reduced with dithionite to obtain a reduced spectrum.

**Collection of Pyridine Hemochrome spectra and Heme Analysis**

For the wildtype or mutants enzymes, 35 µl of the enzyme solution was mixed with an equal volume of 40% pyridine with 200 mM NaOH. The oxidized spectra was measure in the presence of ferricyanide and the reduced in the presence of dithionite. The values of heme b were calculated according to the matrix suggested in (116). The concentration of heme d was estimated using the extinction coefficient \( \varepsilon_{(629-670nm)} = 25 \text{ mM}^{-1}\text{cm}^{-1} \).

**Measurement of oxygen reductase activity**

Oxygen reductase activity was measured using the Mitocell Miniature Respirometer MT200A (Harvard Apparatus). 5 mM DTT and 350 µM Q1 were used as electron donors to measure oxygen reduction by the E445 mutants and wildtype cytochrome bd. 150-250 µM KCN was used to test the cyanide sensitivity of the enzymes.
Structural modelling of cytochrome $bb'$ from *Caldivirga maquilingensis*

Sequences of subunit I from *Geobacillus thermodenitrificans* and *Caldivirga maquilingensis* were aligned using a larger alignment comprising many hundreds of $bb'$ sequences made with the software MUSCLE. This alignment was used as to create a model of subunit I from *Caldivirga* using the *Geobacillus* subunit I as a template on the Swiss Model server. A model of subunit II was also created using subunit I as a template. (The alignments are provided as Supplementary Figures S4 and S5) The model was then visualized using VMD 1.9.2beta1.
### 5.5 Supplementary information

Figure 5.7. Alignments of the S-family cytochrome \( bd \) with other \( bd \)-type oxygen reductases reveals the presence of the motif CxxCH, which binds cytochrome \( c \).

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77
Figure 5.8 Cyanide-insensitive oxygen reduction activity of cytochrome $bb'$. 
Figure 5.9 Mass spectrometric verification of subunit I from Caldivirga cytochrome bd.

Protein sequence coverage: 12%

Matched peptides shown in **bold red**.

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Figure 5.10 Alignment of subunits I from Geobacillus and Caldivirga.

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Figure 5.11 Alignment between subunit II of *Caldivirga* and subunit I from *Geobacillus*.
Chapter 6: References


