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Time-resolved optical absorption spectroscopic studies of cytochrome c oxidase from Rhodobacter sphaeroides and ubiquinol oxidase from Escherichia coli

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TIME-RESOLVED OPTICAL ABSORPTION SPECTROSCOPIC STUDIES
OF CYTOCHROME C OXIDASE FROM RHODOBACTER SPHAEROIDES
AND UBIQUINOL OXIDASE FROM ESCHERICHIA COLI

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

Jennifer Cassano

June 2015

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TIME-RESOLVED OPTICAL ABSORPTION SPECTROSCOPIC STUDIES OF CYTOCHROME C OXIDASE FROM RHODOBACTER SPHAEROIDES AND UBIQUINOL OXIDASE FROM ESCHERICHIA COLI

Jennifer Cassano

Abstract

Knowledge of the mechanism and kinetics of O₂ reduction in heme-copper oxidases (HCOs) is critical for understanding the catalytic functions of these enzymes. To explore this issue, dioxygen reduction in the fully reduced wild-type fusion aa₃ cytochrome c oxidase from Rhodobacter sphaeroides (Rs aa₃) was investigated by multi-wavelength time-resolved optical absorption (TROA) spectroscopy, in combination with the CO flow-flash technique. The reaction of O₂ with the fully reduced E286Q mutant of Rs aa₃, in which a conserved glutamic acid residue was mutated to glutamine, was also investigated. In the wild-type fusion Rs aa₃, the O₂-bound compound Aₚ decayed directly to the oxyferryl intermediate F, while in the bovine heart aa₃ enzyme, compound Aₚ decayed to the so-called Pₚ ("peroxy") intermediate prior to the formation of F. However, in the E286Q Rs aa₃, Pₘ was the terminal intermediate. The detection of Pₚ during O₂ reduction in the bovine heart enzyme but not in the wild-type fusion Rs aa₃ may be due to differences in the relative rates of electron and proton transfer in the two enzymes.

The reactions of O₂ and NO with the fully reduced wild-type fusion Rs aa₃ were investigated in the presence and absence of CO using photolabile O₂- and NO-
complexes and multi-wavelength TROA spectroscopy. The second-order rate constants for O₂ and NO binding in the wild-type fusion Rs aa₃ were the same in both the absence and presence of CO (~1×10⁸ M⁻¹s⁻¹), suggesting that CO does not impede ligand access to the active site in the wild-type fusion Rs aa₃, unlike in the Thermus thermophilus ba₃ (Tt ba₃) enzyme. Moreover, in the absence of CO, O₂ and NO binding in the wild-type fusion Rs aa₃ is 10 times slower than in Tt ba₃ (~1×10⁸ M⁻¹s⁻¹ in Rs aa₃ and ~1×10⁹ M⁻¹s⁻¹ in Tt ba₃). This 10-fold kinetic difference is attributed to structural differences in the proposed ligand channels of Rs aa₃ and Tt ba₃.

The CO photodissociation and rebinding dynamics of the wild-type, G283L, and W172Y/F282T Rs aa₃ enzymes were investigated by TROA spectroscopy. Multiple enzyme conformers of the G283L and W172Y/F282T Rs aa₃ enzymes were observed. Interestingly, in the W172Y/F282T Rs aa₃ enzyme, mutation of the bulky tryptophan and phenylalanine residues in the Rs aa₃ ligand channel to the corresponding smaller residues in Tt ba₃ resulted in ~500 times faster rate of CO recombination compared to that in the wild-type Rs aa₃. The CO was at least partially trapped in the active site cavity of the Rs aa₃ mutants, resulting in the fast CO recombination rate.

The reactions of the mixed-valence wild-type fusion Rs aa₃ and the mixed-valence Escherichia coli bo₃ ubiquinol oxidase (Ec bo₃) with dioxygen were investigated by TROA spectroscopy. In Ec bo₃, F is the intermediate primarily formed upon the decay of compound A in both the fully reduced and mixed-valence
forms of the enzyme. However, in the fully reduced wild-type fusion Rs aa3, compound Ar decays to F, while in the mixed-valence Rs aa3, Am decays to Pm. If a protonable functional group donates a proton during the conversion of P to F in Rs aa3 and Ec bo3, then the detection of Pm as the final intermediate during the reaction of the mixed-valence Rs aa3 with O2 suggests that this functional group may not be protonated in the mixed-valence Rs aa3 enzyme.
Dedication

This thesis is dedicated to my family and friends, for all their support and encouragement. I also dedicate this thesis to my mother—I went all the way in school because of you.
Acknowledgements

First and foremost, I would like to thank my research advisor, Professor Ólöf Einarsdóttir, for her patience and guidance during my graduate studies at UC Santa Cruz. I would also like to thank my committee members, Professor Pradip Mascharak and Professor Roberto Bogomolni, for their guidance throughout the years. I give special thanks to Dr. Istvan Szundi, a senior researcher in our laboratory, for his wisdom and humor. I also want to express my heartfelt thanks to past members of the Einarsdóttir lab, Dr. Chie Funatogawa, Dr. Clive Kittredge, and Dr. William McDonald, for all their help and encouragement.

I want to thank Professor Shelagh Ferguson-Miller (Michigan State University) and Professor Robert Gennis (University of Illinois at Urbana-Champaign) for being generous and supportive collaborators. This thesis would not have been possible without their generous help.

I would also like to thank the Department of Chemistry at UC Santa Cruz. In particular, I want to thank Dr. Eefei Chen, Dr. Randa Roland, Michelle Armstrong, Staci Adams, Samantha Carrington, and Tara deBoer. Thank you to my current and past undergraduate researchers: Manisha Yedla, Maitri Mehta, Terra Villa Gawboy, Clare Liu, Howard Tsoi, Clark Steward, and Katie Edwards.

I want to express my gratitude to Sylvia Choi (University of Illinois at Urbana-Champaign) for teaching me about site-directed mutagenesis. I want to thank
her for her generosity and hospitality during my time in Illinois. She has proven to be an excellent and committed collaborator. I wish her all the best.

I want to thank all my friends who have been there for me. Thank you for listening to me vent and cry. Thank you for giving me the strength and encouragement to persevere. Thank you for helping me through the tough times in my life. I want to give special thanks to all members of the Awana household. Thank you to everyone who helped, pushed, and supported me throughout the years.

Lastly, I want to thank my family—my mother, Wan Yon, my husband, See, and my son, Tristan. Thank you for your love and support.
Chapter 1

Mammalian and Bacterial Heme-Copper Oxidases
General Introduction

1.1 Energy and life

At the cellular level, life exists in a state of disequilibrium. Living cells maintain concentration gradients across membranes and perform numerous reactions that are energetically unfavourable. The Reactome online database (version 50, October 2014) lists 7462 annotated reactions for the human reactome (the set of chemical reactions that may occur within an organism); these reactions are involved in a diverse array of cellular pathways, including apoptosis, regulation of the cell cycle, immune response, DNA replication and repair, and protein metabolism [1-3]. This vast network of chemical activity highlights the state of disequilibrium necessary for life.

For a living cell, metabolic equilibrium represents death and deterioration [4, 5]. A cell at metabolic equilibrium cannot produce the energy needed to drive vital biological activities. The ability to generate energy is therefore fundamental to life. Energy is crucial for processes such as protein synthesis [6, 7], chromosome separation [8, 9], the active transport of ions [10, 11], and muscle contraction [12, 13]. The cell powers these energetically unfavourable processes by coupling them to a reaction which releases a large quantity of energy—namely, the hydrolysis of adenosine triphosphate (ATP) [14].

Hydrolysis of the phosphoanhydride bond of ATP is highly exergonic (Figure 1), releasing 30.5 kJ/mol [14]. Due to its high chemical potential energy, ATP is the primary energy molecule in eukaryotes and most prokaryotes [12]. Cells convert food
Figure 1. The hydrolysis of adenosine triphosphate (ATP) to form adenosine diphosphate (ADP) and inorganic phosphate (P$_i$). Under standard conditions at 25°C and neutral pH, $\Delta G^\circ = -30.5$ kJ/mol [14]. However, the actual free energy change in living cells is much higher, varying from $-50$ to $-65$ kJ/mol, depending upon the intracellular Mg$^{2+}$ concentration [15]. Image prepared using ChemSketch, version 14.01.
into ATP, generating a reservoir of potential energy. The cellular pool of ATP is subsequently used to drive unfavourable biochemical processes. In eukaryotic cells, organelles known as mitochondria execute the transduction of food energy into ATP.

1.2 Mitochondrial structure

Mitochondria are membrane-bound organelles found in most eukaryotic cells (with the exception of red blood cells) [16]. These organelles perform several critical functions, such as the initiation of apoptosis, heme biosynthesis, calcium homeostasis, Fe-S cluster biogenesis, and steroid synthesis [17-21]. However, the primary role of the mitochondrion is ATP synthesis. As such, the mitochondrion has been described as the "powerhouse of the cell" [17, 22]. Mitochondria generate ATP in a process known as oxidative phosphorylation (OX PHOS), whereby a series of respiratory proteins transfer electrons to molecular oxygen, pump protons, and convert ADP to ATP.

Mitochondria are distributed throughout the cytoplasm and occupy a significant portion of space within the cell. Mitochondria occupy as much as 20 to 30% of the cell's volume [23], indicating the importance of energy generation for the cell. The number of mitochondria per cell varies depending upon the cell type, ranging from a few hundred to several thousand per cell [22].

Live cell imaging and time lapse studies have indicated that mitochondria are dynamic—they can move, fuse, and divide within the cell, as well as change their morphology [24, 25]. Mitochondria are typically spherical or oblong, approximately 1
to 4 μm in length, and 1 μm in diameter [22, 26]. At first glance, mitochondria appear
to be similar in size and shape to bacteria, a vestigial fingerprint of the
mitochondrion's endosymbiotic origin\(^1\).

The mitochondrion contains its own highly compact genome, known as
mitochondrial DNA (mtDNA). In mammals, mtDNA is 16.6 bp in length, exists as a
double-stranded circular chromosome, and replicates independently of the cell's
nuclear DNA [34, 35]. Each mitochondrion may contain up to 10 copies of mtDNA
[36]. Because cells contain many mitochondria, the mtDNA copy number per cell can
range from a few hundred to several thousand [22, 37-40]. Mitochondrial DNA
contains genes which encode several transfer RNAs, ribosomal RNAs, and
polypeptides [41]. Mitochondrial DNA codes for subunits I, II, and III of cytochrome
c oxidase, the terminal oxidase of the mammalian respiratory chain; nuclear DNA
encodes the rest of this multisubunit respiratory complex [42].

Figure 2 illustrates key structural features of the mitochondrion: the outer
mitochondrial membrane (OMM), the inner mitochondrial membrane (IMM), the
intermembrane space, and the mitochondrial matrix [26]. The intermembrane space
lies between the outer and inner mitochondrial membranes. The viscous, gel-like

\(^1\) According to the endosymbiont hypothesis, mitochondria arose as an adaptive response to the Great
Oxidation Event (GOE), which occurred approximately 2.4 billion years ago [27-29]. This major
environmental change provoked a dramatic rise in atmospheric oxygen levels. Endosymbiotic theory
proposes that an ancient free-living prokaryote entered the cytoplasm of a primitive eukaryotic host
cell during the GOE [30, 31]. This association gradually developed into a symbiotic relationship: the
host cell provided nutrients, whereas the endosymbiont detoxified oxygen and—via oxidative
phosphorylation—provided energy in the form of ATP [4, 32]. Eventually, the endosymbiont lost its
ability to function independently and became reliant upon the host cell [30, 31, 33]. The proto-
eukaryotic cell retained the endosymbiont for its oxidative phosphorylation system, thereby giving rise
to present day mitochondria.
Figure 2. "Baffle" model of a mitochondrion. The mitochondrion contains two distinct membranes: the outer mitochondrial membrane and the inner mitochondrial membrane. The intermembrane space is the region between the inner and outer mitochondrial membranes. The inner mitochondrial membrane encloses the mitochondrial matrix. Image adapted from: http://micro.magnet.fsu.edu/cells/mitochondria/mitochondria.html. Michael W. Davidson and Florida State University.
matrix is enclosed by the IMM and contains a concentrated mixture of proteins, mtDNA, tRNAs, and ribosomes [43].

The OMM is a smooth, curved surface in contact with the cytoplasm of the cell. The OMM completely encapsulates the mitochondrion, serving as the outer boundary. The outer membrane is interspersed with pore-forming proteins, enabling small metabolites and ions to freely permeate through the membrane [43].

The IMM, in contrast, is highly impermeable; specific membrane transport proteins are required to convey molecules and ions across the IMM [26, 44]. The IMM is characterized by an intricate, convoluted architecture which maximizes the membrane surface area available for biochemical reactions. The IMM is extremely crowded with proteins, exhibiting a 75:25 ratio of protein to lipid [23, 45, 46].

Proteins of the OX PHOS system reside in the IMM. The IMM is so protein-dense that the reactions of the OX PHOS system rely on frequent collisions between protein complexes rather than the existence of an organized linear chain or assembly line.

The "baffle" model paradigm states that the IMM folds in upon itself, creating structures known as cristae. However, electron tomography now lends support for the cristae-junction model (Figure 3), which proposes that the IMM contains two distinct domains: an inner boundary membrane (IBM) and a cristae membrane (CM) [22, 45]. The IBM and CM are joined together via narrow, tubular connections called cristae junctions [47, 48]. The IBM is enriched with proteins involved with the import of mitochondrial proteins [26]. The OX PHOS complexes are assembled in the IBM before they are shuttled to the CM. Immunolabeling and electron microscopy studies
Figure 3. Cristae junction model of a HeLa cell mitochondrion, as illustrated by 3D electron tomography. The views rotate about a central axis and show the outer membrane (translucent blue), the inner boundary membrane (white), and cristae (various colors). Results from electron tomography have caused a paradigm shift from the "baffle" model to the cristae junction model. Image from reference [49].
have shown that respiratory chain proteins are primarily concentrated in the CM [47], indicating that the CM is the principal site for oxidative phosphorylation.

1.3 The mitochondrial respiratory chain

The metabolism of food produces NADH and FADH₂, both of which are cofactors capable of transferring electrons between redox centers. Electrons from NADH and FADH₂ enter the mitochondrial respiratory chain, whereupon they pass through a series of redox centers until they ultimately reduce dioxygen to water [47]. The redox reactions of the respiratory chain release energy, which is utilized to pump protons across the IMM, from the matrix to the intermembrane space. The pumping of protons establishes voltage and proton gradients across the IMM, giving rise to a proton motive force (pmf). The pmf then drives the energetically unfavorable phosphorylation of ADP to ATP.

Five protein complexes comprise the mitochondrial respiratory chain (Figure 4), also known as the electron transport chain or OX PHOS system. Table 1 summarizes key features of the respiratory complexes, and Table 2 summarizes the reactions. Complex I (NADH:ubiquinone oxidoreductase) extracts two electrons from NADH, donates them to ubiquinone (reducing it to ubiquinol), and pumps protons across the IMM [50]. Complex II (succinate dehydrogenase; alternatively called succinate:ubiquinone oxidoreductase) functions in both the citric acid cycle and the respiratory chain [51]. Complex II, which does not contribute to the pmf, couples the oxidation of succinate to fumarate with the reduction of FAD to FADH₂. Complex II
**Figure 4.** The mitochondrial electron transport chain. Complex I is shown in blue, Complex II in green, Complex III in red, cytochrome c in yellow, Complex IV in cyan, and ATP synthase in purple. (PDB files 3M9S, 3AEF, 1BGY, 1OCC, 1J3S, and 4B2Q). Image made using Pymol.
<table>
<thead>
<tr>
<th>Complex</th>
<th>No. of subunits</th>
<th>Molecular mass</th>
<th>Cofactors present</th>
<th>Protons pumped</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>45</td>
<td>1 MDa</td>
<td>8 Fe-S, 1 FMN</td>
<td>4 H⁺ / 2 e⁻</td>
<td>[41, 52-54]</td>
</tr>
<tr>
<td>Complex II</td>
<td>4</td>
<td>124 kDa</td>
<td>1 FAD, 3 Fe-S heme b</td>
<td>None</td>
<td>[41, 55-57]</td>
</tr>
<tr>
<td>Complex III</td>
<td>11</td>
<td>240 kDa</td>
<td>1 FeS, heme bₐ, heme bₜ, heme c₁</td>
<td>1 H⁺ / 1 e⁻</td>
<td>[41, 58, 59]</td>
</tr>
<tr>
<td>Complex IV</td>
<td>13</td>
<td>200 kDa</td>
<td>Cuₐ, heme aₐ, heme aₐ, Cuₐ</td>
<td>1 H⁺ / 1 e⁻</td>
<td>[41, 60, 61]</td>
</tr>
<tr>
<td>ATP synthase</td>
<td>16</td>
<td>560 kDa</td>
<td>None</td>
<td>3 - 4 H⁺ per e⁻</td>
<td>[41, 62, 63]</td>
</tr>
</tbody>
</table>

Table 1. Summary of the mitochondrial aerobic respiratory chain.
<table>
<thead>
<tr>
<th>Complex</th>
<th>Reaction catalyzed</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>( \text{NADH} + Q + 5\text{H}^+_{\text{in}} \rightarrow \text{NAD}^+ + \text{QH}<em>2 + 4 \text{H}^+</em>{\text{out}} )</td>
<td>[64]</td>
</tr>
<tr>
<td>Complex II</td>
<td>( \text{succinate} + Q \rightarrow \text{fumarate} + \text{QH}_2 )</td>
<td>[56]</td>
</tr>
<tr>
<td>Complex III</td>
<td>( \text{QH}<em>2 + 2 \text{cyt c}</em>{\text{oxid}} + 2 \text{H}^+<em>{\text{in}} \rightarrow Q + \text{cyt c}</em>{\text{red}} + 4 \text{H}^+_{\text{out}} )</td>
<td>[58]</td>
</tr>
<tr>
<td>Complex IV</td>
<td>( 4 \text{cyt c}<em>{\text{red}}^{2+} + 8 \text{H}^+</em>{\text{in}} + \text{O}<em>2 \rightarrow 4 \text{cyt c}</em>{\text{oxid}}^{3+} + 2 \text{H}<em>2\text{O} + 4 \text{H}^+</em>{\text{out}} )</td>
<td>[65]</td>
</tr>
<tr>
<td>ATP synthase</td>
<td>( \text{ADP} + \text{P}<em>i + n\text{H}^+</em>{\text{in}} \rightarrow \text{ATP} + n\text{H}^+_{\text{out}} )</td>
<td>[15]</td>
</tr>
</tbody>
</table>

**Table 2.** Reactions catalyzed by the mitochondrial respiratory complexes.
subsequently transfers electrons from FADH$_2$ to ubiquinone [55]. Complex III (cytochrome $c$ reductase; alternatively called the $bc_1$ complex) catalyzes the transfer of two electrons from ubiquinol to two molecules of cytochrome $c$, a water-soluble protein capable of carrying electrons. Complex III also contributes to the pmf by pumping protons into the intermembrane space. Complex IV (cytochrome $c$ oxidase) accepts electrons from cytochrome $c$ in order to reduce dioxygen to water, pumping protons across the IMM in the process [55]. The next section discusses the structure and proposed O$_2$ reduction mechanism of cytochrome $c$ oxidase in greater detail. The fifth member of the OX PHOS system is ATP synthase, which utilizes the pmf to drive ATP synthesis.

1.4 Molecular oxygen and the importance of cytochrome $c$ oxidase (Complex IV)

Atmospheric oxygen is relatively stable, despite the fact that the oxidation of organic molecules is thermodynamically favorable. The most abundant and stable form of dioxygen exists in the triplet ground state, which contains two unpaired electrons that: 1) occupy degenerate molecular orbitals, and 2) have parallel spins [66, 67]. In contrast, organic molecules predominantly exist in the singlet state with spin-paired electrons. Spin-restriction rules prohibit the direct reaction of triplet oxygen with singlet molecules [66, 68]. The electronic configuration of atmospheric O$_2$ essentially creates an activation barrier that prevents dioxygen from spontaneously oxidizing organic compounds. Furthermore, uncontrolled oxygen chemistry can also
produce cytotoxic reactive oxygen species, such as superoxide (O$_2^*$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (OH$^*$) [67].

Nature has evolved metalloenzymes, such as cytochrome $c$ oxidase and other heme-copper oxidases, which can activate dioxygen and catalyze its reduction to water [67]. CcO accomplishes this task without the formation of toxic reactive oxygen species. Approximately 90 to 95% of the oxygen taken up by the cell binds to the active site of CcO, where the enzyme reduces $O_2$ to H$_2$O [69, 70]. Without CcO, the electron transport chain would become disrupted, compromising ATP production.

CcO performs three essential functions: it oxidizes molecules of cytochrome $c$, catalyzes the reduction of dioxygen to water, and pumps protons across the IMM. The reduction of $O_2$ to water releases a large amount of energy, with a $\Delta G$ of -231.8 kJ/mol [55]. CcO couples this exergonic process with the endergonic translocation of protons across the inner mitochondrial membrane [71]. During catalysis, eight protons are taken up on the matrix side (N-side) of the membrane, and four protons are pumped into the intermembrane space (P-side). The remaining four protons are used in dioxygen reduction. One proton is pumped per electron. The process is summarized in the following reaction:

$$4 \text{ cyt} \text{ } c_{\text{red}}^{2+} + 8 \text{ H}^+_{\text{in}} + \text{O}_2 \rightarrow 4 \text{ cyt} \text{ } c_{\text{oxid}}^{3+} + 2 \text{ H}_2\text{O} + 4 \text{ H}^+_{\text{out}}$$

Deficiencies in CcO have been associated with numerous disease phenotypes, including necrotic lesions in the brain (Leigh syndrome), hypertrophic cardiomyopathy (thickening of the heart muscle, causing the heart to work harder), fatal infantile lactic acidosis (buildup of lactic acid in the urine, cerebrospinal fluid,
and blood), hepatic failure, encephalopathy (severe brain dysfunction), Leber's hereditary optical neuropathy, and sideroblastic anemia [72-75]. Individuals afflicted with CcO deficiencies tend to exhibit decreased levels of CcO enzyme activity [76]. CcO dysfunction may arise due to mutations in the mtDNA-encoded genes for CcO or mutations in the nuclear DNA, which encode proteins necessary for the proper assembly of CcO [77]. Mutations in the mtDNA-encoded subunit of CcO have been associated with colon cancer [34] and prostate cancer [78, 79]. Highly respiring cells, such as brain, muscle, and heart cells, appear to be especially sensitive to CcO defects. Researchers have suggested that a malfunctioning OX PHOS system may cause cell death by limiting the energy available to the cell.

Of the mitochondria-related diseases, CcO deficits are among the most common in childhood [72]. CcO deficiencies frequently exhibit a very early onset; many disorders manifest themselves in infancy or early childhood, although examples of later onset have been reported. Depending upon the severity of the illness, patients may display profound cognitive and motor delays, muscle weakness, poor muscle tone (hypotonia), and failure to gain weight and grow. Disease progression is often swift and debilitating and, in many cases, fatal within a span of several years.

Aside from its biological and medical significance, CcO is also important due to its potential use in biofuel cell (BFC) technology. There is considerable interest in designing BFCs capable of converting chemical energy into electrical energy using enzymes and biological substrates within living organisms [80-83]. Many BFCs have employed glucose and O2, both of which are found within physiological fluids and
tissues; these biofuel cells produce electrical energy by oxidizing glucose at the anode and reducing dioxygen at the cathode [83]. CcO serves as a logical choice for use at the cathode, and Katz et al. [84] constructed two biofuel cells, with CcO as the reductive catalyst in both cases. The use of CcO as a biocatalyst is attractive due to its ability to function at neutral pH and ambient temperature. In addition, CcO-immobilized electrodes have been investigated for potential use in biofuel cells [85, 86]. BFCs utilizing endogenous substrates such as glucose and O₂ may serve as power sources for implantable medical devices, including biosensors, micro-pumps, or pacemakers [82, 83, 87].

1.5 The heme-copper oxidase superfamily

Cytochrome c oxidase belongs to the heme-copper oxidase (HCO) superfamily of enzymes. Enzymes of this family are membrane-bound proteins, and are found in all three domains of life: Eukaryota, Bacteria, and Archaea [88]. The HCO superfamily is divided into two classes: 1) heme-copper oxidases (also called oxygen reductases), which reduce dioxygen to water during aerobic respiration, and 2) nitric oxide reductases, which convert NO to N₂O. The oxygen reductases are further subdivided into three families: A-, B-, and C-families based upon sequence and structural evidence; up to eight families have been proposed elsewhere [89].

Heme-copper oxidases vary with regard to the identity of the electron donor they interact with (such as cytochrome c or ubiquinol) and the number of protein subunits present [88]. HCOs also vary in the type of heme groups present within the
enzyme, such as heme A, O, B, or C (Figure 5) [65]. Although there is considerable structural and genetic diversity among the HCOs, they all share certain basic elements: the presence of multiple heme groups (one of which forms part of the enzyme active site) and one or more copper sites. All members contain a redox-active heme iron-copper binuclear center (BNC) [90, 91] that serves as the catalytic site, where the \( \text{O}_2 \) reduction chemistry takes place. The BNC contains a penta-coordinate heme Fe located in close proximity to a copper atom (Cu\(_B\)). A histidine residue occupies the proximal coordination site of the BNC iron, and three histidine residues are coordinated to Cu\(_B\).

1.6 The structures of bovine heart aa\(_3\) and Rhodobacter sphaeroides aa\(_3\) cytochrome \( c \) oxidases and Escherichia coli bo\(_3\) ubiquinol oxidase

**Bovine heart aa\(_3\) cytochrome \( c \) oxidase**

X-ray crystallography has resolved structures of CcO from several organisms, including *bos taurus*, *Rhodobacter sphaeroides*, and *Paracoccus denitrificans* [60, 92, 93]. However, CcO from bovine heart has been studied the most intensively. Figure 6 illustrates the X-ray crystal structure of bovine CcO, which crystallizes as a dimer. Bovine CcO is a complex assembly of 13 polypeptide subunits, with a total molecular weight of approximately 200 kDa [94, 95]. The bovine enzyme belongs to the A-family of HCOs and shares high sequence homology (98%) with human CcO. Mitochondrial DNA encodes for subunits I, II, and III; nuclear DNA encodes for the remaining ten subunits [96]. Subunits I through III are referred to as the core subunits,
Figure 5. Structures of heme A, O, B, and C. Heme A contains a hydroxyethyl farnesyl chain and a formyl group. The structure of heme O is identical to that of heme A, except heme O has a methyl group instead of a formyl group. Heme B contains a vinyl and a methyl group. Heme C is similar to heme B, however, heme C is covalently bound to the polypeptide via thioether linkages. Images prepared using ChemSketch, version 14.01.
**Figure 6.** The 13 subunit bovine heart cytochrome c oxidase complex. PDB 2EIJ.

Image created using Pymol.
because these three subunits are essential for catalytic turnover. Subunits I and II are necessary because they contain the redox-active metal centers; however, subunit III is also critical in preventing turnover-induced inactivation of the enzyme (termed "suicide inactivation") [97].

Figure 7 shows the four redox-active metal centers in bovine CcO: heme $a$, heme $a_3$, Cu$_A$, and Cu$_B$. A bimetallic Cu-Cu site, denoted as Cu$_A$, is present in subunit II and functions as the initial acceptor of electrons from cytochrome $c$. The fully oxidized Cu$_A$ site is valence delocalized, with +1.5 assigned to each copper [98]. Two cysteine residues (Cys 196 and Cys 200) bridge the copper ions. Furthermore, methionine (Met 207), glutamate (Glu 198), and two histidine residues (His 161 and His 204) are ligated to the Cu$_A$ site such that each copper ion is tetra-coordinate.

Bovine CcO contains two heme A prosthetic groups, both of which are found in subunit I [60]. Heme A is characterized by the presence of a hydroxyethyl farnesyl side chain and formyl group attached to the iron porphyrin macrocycle [99]. The electron-withdrawing formyl group is responsible for the vibrant green color of the fully reduced enzyme. One of the hemes, denoted heme $a$, is ligated by two histidine residues (His 61 and His 378). Heme $a$ contains a six-coordinate, low-spin iron which participates in electron transfer processes. Because heme $a$ is coordinatively saturated, it is incapable of ligand binding. The remaining heme, designated as heme $a_3$, contains a penta-coordinate, high-spin iron. Heme $a_3$ is coordinated to an axial histidine (His 376), with the other axial position open to binding small molecules such as CO, NO, CN$^-$, and O$_2$ [65]. Heme $a_3$ forms part of the binuclear active site.
**Figure 7.** The redox-active metal centers of bovine heart cytochrome $c$ oxidase (PDB 2EIJ). Top panel: the bimetallic Cu$_A$ site. Middle panel: hexa-coordinate low-spin heme $a$. Bottom panel: the binuclear center, comprised of high-spin heme $a_3$ and Cu$_B$. Images created using Pymol.
along with a copper ion, denoted as CuB. The binuclear center is housed within subunit I and serves as the catalytic site of dioxygen reduction to water.

CuB is located in close proximity to the heme $a_3$ iron, approximately 4.8 Å away. CuB is ligated to three histidine residues, His 240, His 290, and His 291. One of these histidine residues, His 240, forms a covalent cross-link with a nearby tyrosine residue, Tyr 244 [60]; this cross-link represents a post-translational modification which is highly conserved among the bacterial heme-copper oxidases. The OH group of Tyr 244 faces the active site and is hydrogen bonded to the hydroxyethyl farnesyl side chain of heme $a_3$. The exact role of the Tyr 244 hydroxyl group is unknown, however, it has been suggested that the OH group donates an electron and/or H$^+$ during catalysis [100]. Mutation of Tyr 244 to phenylalanine abolished enzyme activity [101], indicating that the Tyr-His cross-link plays a significant role in the function and structure in CcO.

Figure 8 shows the distances between the metal centers in bovine CcO. Heme $a_3$ is located within 13.1 Å of heme $a$, whereas CuA lies within approximately 19.5 Å of heme $a$ [60]. Upon binding to CcO, molecules of cytochrome $c$ transfer electrons one at a time to CuA [65]. Electrons are then transferred from CuA to heme $a$, and, finally, to the binuclear center, where dioxygen reduction takes place. Section 1.7 will focus on the mechanism of dioxygen reduction. The pathway of electron transfer is as follows: cytochrome $c \rightarrow$ CuA $\rightarrow$ heme $a \rightarrow$ binuclear center (Figure 9).

Two additional non-redox metals are tightly associated with the bovine enzyme: A Mg$^{2+}$ binding site is present at the interface between subunits I and II, and
Figure 8. Distances between the redox-active metal centers in bovine heart cytochrome c oxidase. PDB 2EIJ. Image created using Pymol.
Figure 9. Proposed electron transfer pathway in $aa_3$-type oxidases. PDB 2EIJ. Image created using Pymol.
a Zn²⁺ ion is located within the nuclear DNA-encoded subunit Vb, on the matrix side of the membrane [102-104]. A Ca²⁺ ion has also been detected in bovine heart CcO [60, 102]. The magnesium and calcium ions may be important for the structural stabilization of the enzyme. The functional role played by the zinc ion remains unclear.

**Rhodobacter sphaeroides aa₃ cytochrome c oxidase**

Because each mammalian cell potentially contains thousands of mtDNA copies [22, 105], it is challenging to conduct mutagenesis studies on the mtDNA-encoded core subunits of CcO. Thus, many researchers have elected to use bacterial oxidases, which are relatively simple and more amenable to genetic manipulation than the mammalian enzyme. Bacterial oxidases are encoded by nuclear DNA and typically contain three or four protein subunits, making them less complex than the 13-subunit bovine enzyme. Furthermore, site-directed mutagenesis techniques are routinely used to investigate the structural and functional roles of amino acids in bacterial oxidases. Mutational studies on cytochrome c oxidase frequently employ the bacterium *Rhodobacter sphaeroides* [106].

*Rhodobacter sphaeroides* is a Gram-negative α-proteobacterium found in eutrophic lakes and ponds (Figure 10) [107-109]. *R. sphaeroides* is a metabolically versatile organism capable of photosynthesis, fermentation, anaerobic respiration, and aerobic respiration [108-111]. *R. sphaeroides* can tolerate anoxic as well as oxic conditions, and is able to fix carbon and nitrogen. In the light, *R. sphaeroides* can
Figure 10. Transmission electron micrograph of *R. sphaeroides*. Image from reference [112].
function as a photoheterotroph or photoautotroph; however, in the dark, it can grow
chemoautotrophically or chemoheterotrophically. These diverse metabolic pathways
enable *R. sphaeroides* to adapt to changes in light intensity, oxygen availability, and
nutrient sources in the environment [108, 109].

Under aerobic conditions, *R. sphaeroides* expresses a branched respiratory
chain that includes two NADH:ubiquinone oxidoreductases, succinate dehydrogenase,
the $bC_1$ complex, two species of cytochrome $c$ (water-soluble $c_2$ and lipid-soluble $c_y$),
two cytochrome $c$ oxidases$^2$ ($aa_3$ and $cbb_3$), and a $bd$-type ubiquinol oxidase$^3$ [108,
111, 115, 116]. The $aa_3$-type cytochrome $c$ oxidase (CcO) predominates when *R.
sphaeroides* grows aerobically in the dark, whereas the $cbb_3$ oxidase is expressed
under photosynthetic conditions or low oxygen tension [115]. Expression of the $bd$
oxidase is upregulated under anoxic conditions (extremely low levels of dissolved
oxygen).

The $aa_3$-type CcO from *R. sphaeroides* ($Rs aa_3$) is a four subunit enzyme
with a combined molecular mass of approximately 130 kDa [92, 113]. Subunits I, II,
and III are homologous to the core subunits of bovine CcO. However, subunit IV of
the bacterial CcO shares no homology with any of the bovine subunits, and its
functional or structural role is unknown. The primary amino acid sequence of $Rs$ CcO
subunit I is 55% identical to bovine subunit I; $Rs aa_3$ subunit II is 39% identical to

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$^2$ Genes which code for a $caaa_3$-type cytochrome $c$ oxidase have been identified in the *R. sphaeroides*
genome. However, expression of this putative $caaa_3$ oxidase in *R. sphaeroides* has not been observed
yet [111, 113, 114].

$^3$ The $goxA$AB operon of *R. sphaeroides* encodes for a quinol oxidase. However, expression of this
putative quinol oxidase has not been observed yet [111, 114].
bovine subunit II; and *Rs aa* subunit III is 49% identical to bovine subunit III (Figures 11 through 13).

Figure 14 illustrates the crystal structure of *Rs CcO* [92]. *Rs CcO* spans the bacterial plasma membrane and couples the four electron reduction of dioxygen to the translocation of protons across the plasma membrane. Subunit I consists of 12 transmembrane helices and contains heme *a* as well as the active site constituents, heme *a*₃ and Cuₜ. Furthermore, crystallographic studies have identified two proton pathways in subunit I which transport H⁺ to the binuclear center and across the IMM. These pathways are lined with residues which coordinate highly ordered water molecules. The D-pathway contains a highly conserved aspartate (D132), and the K-pathway contains a conserved lysine (K362) [117, 118].

Subunit II is comprised of two transmembrane helices and a globular domain that lies outside of the plasma membrane [92]. The globular domain is a ten stranded β-barrel which contains the Cuₐ site near the C-terminal end [119]. In addition, the globular domain, located in the bacterial periplasm, contains a binding site for the mobile electron carrier cytochrome *c*; this docking site is lined with negatively charged residues and a conserved tryptophan, which facilitates electron transfer from cytochrome *c* to the Cuₐ site [92].

Subunit III is composed of seven transmembrane helices and makes extensive contacts with subunit I [92]. A V-shaped cleft organizes subunit III into two bundles, with two α-helices for the N-terminal bundle, and five α-helices for the C-terminal bundle. The N-terminus contains three conserved histidine residues ~10 Å away from
**Bov aa**3 SI  M------------ -----FINRW LFSTNHKDIG TLYLLFGAWA GMVGTALSLL
**Rs aa**3 SI  MADAAIHGHE HDRRGFFTRW FMSTNHKDIG VLYLFTGGLV GLLSVAPTVY

**Bov aa**3 SI  IRAELGQPQTL LL---------- G---------- --------- DDQIYNVVVT
**Rs aa**3 SI  MRMELMAPGQ QFMCAEHLAS GLVKGFFQSL WPSAVENCQP NGHLMNVMIT

**Bov aa**3 SI  AHAFVMIFFFM VMPIMIGGFG NWLVPLMIGA PDMAPRMMNN MSFWLPPPSF
**Rs aa**3 SI  GHGILMFFFP VPALFGGGFG NYFMPMLHIGA PDMAPRMMNN LSFWLVLGAT

**Bov aa**3 SI  LLLLASSMV- -----EAGAGT GWTNYPPLAG NLHAHASVD LTIFSFLHLAG
**Rs aa**3 SI  SLAVASLFAP GGNGQLGSGI GWVLYPPPLA STSESQSTLD LAIFAVHLASG

**Bov aa**3 SI  VSSILGAINF ITTTIINMKPP AMSQYQTPLF VWSVMITAVL LLLSLPVLAA
**Rs aa**3 SI  ASSILGAINM ITTFNLNMRAP GMTMKVPLF AYSIFVTAWL ILLALPVLAG

**Bov aa**3 SI  SHIVTYSGK KEPFPGYMGV WAMMSIGFLG FIVWAHHMFT VGMDVDRTRAY
**Rs aa**3 SI  SHVIATFAP-K KPIFGYLPMV YAMVASIGVLG FVWAAHMYMT ALLLSSTQSY

**Bov aa**3 SI  LNDTWA-KIH FAIMFVGVNM ATLHGGNIKW SPAMMWALGF IFLFTVGGYL
**Rs aa**3 SI  SSLGAFLSFA SFLFFLGWIF YTLTRGARVT ANNYWNEHAD TLEWLTLTSP

**Bov aa**3 SI  PYHTFEE-PT YVNLK-----
**Rs aa**3 SI  PEHTFQLPK REDWERAPAH

**Figure 11.** Subunit I amino acid sequence alignment of the cytochrome c oxidases from *R. sphaeroides* and bovine heart.
**Figure 12.** Subunit II amino acid sequence alignment of the cytochrome c oxidases from *R. sphaeroides* and bovine heart.
Figure 13. Subunit III amino acid sequence alignment of the cytochrome c oxidases from *R. sphaeroides* and bovine heart.
Figure 14. X-ray crystal structure of *R. sphaeroides* cytochrome *c* oxidase (PDB 1M56). Image created using Pymol.
the entrance to the D-pathway. Mutation of these His residues suggests they may play a role in facilitating proton uptake into the D-channel [123]. Although subunit III contains no redox centers, it is important for the stability of CcO. When subunit III is removed, the enzyme undergoes turnover-induced inactivation. This phenomenon, which occurs by an unknown mechanism, causes the spontaneous and irreversible inactivation of the enzyme during catalytic turnover [120]. In the absence of subunit III, proton pumping is decreased, the redox potential of heme $a_3$ is decreased, and Cu$_B$ is partially lost [97]. Furthermore, multiple phosphatidylethanolamine molecules are tightly associated with subunit III. Removal of these lipids also results in turnover-induced inactivation. Thus, it has been suggested that subunit III prevents turnover-induced inactivation by maintaining the structural stability of CcO.

Subunit IV is the smallest subunit of Rs CcO, consisting of a single membrane-spanning helix [92]. This subunit shares no homology to any of the mitochondrial subunits, and its function is currently unclear.

Subunits I, II, and II of Rs $aa_3$ are essential for catalysis, and form the catalytic core of the bacterial enzyme. Remarkably, Rs $aa_3$ has significant sequence and structural homology to the mitochondrial $aa_3$-type cytochrome $c$ oxidase in eukaryotes (Figure 15) [60, 92]. High homology and simpler subunit pattern have made R. sphaeroides an important model system for investigating proton pumping and dioxygen reduction in cytochrome $c$ oxidase [106].
Figure 15. Structural alignment of the core subunits (I, II, and III) of the cytochrome c oxidases from bovine heart (PDB 2EIJ, cyan) and *R. sphaeroides* (PDB 1M56, green). Structure alignment performed using Pymol.
*Escherichia coli* bo$_3$ ubiquinol oxidase

*Escherichia coli* is a Gram-negative bacterium capable of generating energy via aerobic respiration, anaerobic respiration, or fermentation [121]. This metabolic flexibility enables *E. coli* to adapt to changes in its environment. Due to the ease with which its genome can be manipulated, *E. coli* is routinely used in molecular biology as a tool for creating gene constructs and expressing recombinant proteins [122].

In the presence of oxygen, the aerobic respiratory chain of *E. coli* is branched, containing numerous dehydrogenases, a quinone pool (consisting of ubiquinone, menaquinone, and demethylmenaquinone), and three quinol oxidases (*bo$_3$, bd-I, and bd-II) [123, 124]. Organic substrates, such as NADH, succinate, lactate, and pyruvate, donate electrons to the final electron acceptor, molecular oxygen; the transfer of electrons is mediated by the dehydrogenases, quinones, and quinol oxidases present within the plasma membrane of *E. coli*. The bo$_3$ oxidase is expressed when oxygen levels are high, whereas the bd-I oxidase is expressed when oxygen is limited [125]. The physiological role of the bd-II oxidase is unclear.

Cytochrome bo$_3$ ubiquinol oxidase from *E. coli* (*Ec bo$_3$*) accepts two electrons from ubiquinol-8 (which contains eight isoprenoid units), catalyzes the reduction of dioxygen to water, and pumps protons across the bacterial plasma membrane (Figure 16) [126, 127]. The transmembrane electrochemical proton gradient may then be utilized to power energetically unfavorable processes within the cell. *Ec bo$_3$* is a four subunit enzyme comprised of twenty-five transmembrane helices, with a combined molecular mass of ~130 kDa [130]. *Ec bo$_3$* is a member of the A-family of heme-
Figure 16. X-ray crystal structure of bo$_3$ ubiquinol oxidase from E. coli (PDB 1FFT).

Image created using Pymol.
copper oxidases, and is structurally very similar to the Rs aa3 enzyme—indeed, the
crystal structures of Ec bo3 and Rs aa3 are superimposeable (Figure 17) [92, 127].

However, despite having similar protein architectures, there are key
differences between the Ec bo3 and Rs aa3 enzymes. In Rs aa3, cytochrome c
functions as a water-soluble electron carrier, delivering electrons one at a time to the
enzyme [128]. However, in Ec bo3, the membrane-soluble ubiquinol-8 serves as the
electron carrier, and is capable of delivering two electrons to the bo3 oxidase [113].
Furthermore, Ec bo3 contains a low-spin heme b and a binuclear active site consisting
of CuB and Fe from high-spin heme o3 [127]. The dinuclear CuA site, found in Rs aa3
subunit II, is altogether lacking in Ec bo3. It is hypothesized that heme b receives
electrons from ubiquinol-8, in a putative mechanism involving two ubiquinol-8
binding sites—a high affinity QH site, and a low affinity QL site [129]. The prevailing
theory suggests that a molecule of ubiquinol-8 binds tightly at the QH site, and serves
to mediate electron transfer from the substrate ubiquinol-8, which is bound to the QL
site [129]. The exact locations of the QL and QH sites are still a matter of debate. In
this proposed mechanism, the path of electron transfer is as follows: QL site → QH
site → heme b → heme o3–CuB binuclear center.

Subunit I of Ec bo3 is comprised of 15 membrane-spanning helices, and is the
largest of the subunits [127]. All three redox-active metal centers are contained within
subunit I. The amino acid sequences of subunit I from Ec bo3 and Rs aa3 are ~30%
identical (Figures 18). The post-translational tyrosine-histidine crosslink, seen in the
bovine and Rs aa3 enzymes, is not visualized in the crystal structure of Ec bo3 due to
Figure 17. Structural alignment of the core subunits (I, II, and III) of *R. sphaeroides* aa$_3$ cytochrome c oxidase (PDB 1M56, green) and *E. coli* bo$_3$ ubiquinol oxidase (PDB 1FFT, blue). Structure alignment performed using Pymol.
Rs aa3 SI  MADAAAAAI HGHEHAAAAA ------D RRGFFTAAAAA ------RWFM
Ec bo3 SI  MFGLSLSDAII PFPHEPVIMT IAIGILGGLA LVGLITYFGK WTYLYKEHWE LT

Rs aa3 SI  STNHDKIDIGVL YLFTGGLVLG ISVAFTVYMRF MEMLAPGQVF MCAEHLESGL
Ec bo3 SI  SVDHHKRLGIM YIIVAIYMLL RGFADAIMMR SQ-------- ---QALASAG

Rs aa3 SI  VKGFFQSLWP SAVENCPTNG HLWNVMTMGH GILMMPVIVVI PALFGGFNY
Ec bo3 SI  EAGFAAAAA LPP AAAA AA HHYDQIFTAH GVEIMFFVAM PFVIG- LMNL

Rs aa3 SI  PMPLHIGAPD MAFFMRNLS YWLTYAGTSL AVASLFAPGQ NGQLGGSGWG
Ec bo3 SI  VVPLQIGARD VAFPPFLNLIS FWFTVGLV NWNL--------G VGEFAQ--TG W

Rs aa3 SI  VLYPPSLTSE --SGYSTLDA IFHAVHLSGAS SIILGAINMNT TFLNMRAPGM
Ec bo3 SI  LAYPLSGIE YSPVGVDYW IWSLQSGIG TTTGINFFV TILMRAPGM

Rs aa3 SI  TMHKVPLFAW SIFVTAWLIL LALPVLAGAI TMLLDRNFIF TTFQQPPSGG
Ec bo3 SI  TMFKMPVFTW AILCANDVLI ASFPILTVTV ATLLDLRYS LGFHFTDMMG

Rs aa3 SI  DPVLQYHILW FFGHPEVYII VLPAPFGLSY VIATFakkPI FGFLPMVAM
Ec bo3 SI  NMMEYINLWL AWGHPEVYIL ILPVFGVFSE IAATFSRKRL FGYSLSVWAT

Rs aa3 SI  VAIGVLGFVV WAHHMTAGL SLTQSSYFMM ATMVIAVPTG IKIFSWIATM
Ec bo3 SI  VICVTLFSIFV WLYHFPMTGMA GANVNAFFGI TTMIAATPG VKIFWNLFTM

Rs aa3 SI  WGGSLIEKTP MLWALGFLFL FTWGVTGIVL LSQASVDRYY HDTYVVAHF
Ec bo3 SI  YQGRIVFHS AMLWVIIFGTV ATVGGMTGVL LAVPGDFVL HNLVFLIAHF

Rs aa3 SI  HYVMSLGAVF GIFAGIYFWI GKMSPGQYPE WAGKLFHWMM FVGNALTFFP
Ec bo3 SI  HNVIIGGVFV GCFGQMGTF PWAFGFKLNE TWGKRAFWFV IIGFFVAPMP

Rs aa3 SI  QHFLGRQGMP RRRY---IDYP EAFATWNVFS SLGAPLSTFAS FLFFLVGFY
Ec bo3 SI  LYALGFMGMT RRLSQQID-P Q-FHTMLMIA ASGAVL-IAL GILCLVQMY

Rs aa3 SI  TLTRGAAAAA AR VTTANYWNEH ADTLEWTLTS PPPEHTFEQL PKREDAAAAAA W
Ec bo3 SI  VSIRDRDQNRR DLTGDHPQW-- GRTELATSS PFPYFNPQVW PVVHERDAFW

Rs aa3 SI  E----------- APAH---------------- ------------------------
Ec bo3 SI  EMKEKGEAYK KPDHYEEIIHM PKNSGAGIVI AAFSTIGFA MIWHIWWLAI

Rs aa3 SI  ---------------- -------------------------- -------------------
Ec bo3 SI  VGFAGMIIITW IVKSPFEDVD YVYVPAEIEK LENQHFDEIT KAGLKNAG

Figure 18. Subunit I amino acid sequence alignment of R. sphaeroides aa3
cytochrome c oxidase and E. coli bo3 ubiquinol oxidase.
its limited resolution. Subunit II from *Ec bo*₃ is effectively divided into two domains: an N-terminal domain and a C-terminal domain. The N-terminal domain is anchored within the membrane via two transmembrane helices, and the C-terminal domain extends into the periplasm. Subunit II is almost exclusively in contact with subunit I. The amino acid sequence of subunit II from *Ec bo*₃ is ~19% identical to that of *Rs aa*₃ (Figure 19). Subunit III consists of five transmembrane helices, which are in contact with subunits I and IV. The amino acid sequences of subunit III from *Ec bo*₃ and *Rs aa*₃ are ~23% identical to each other (Figure 20). The smallest subunit is subunit IV, which makes contacts with subunits I and III.

1.7 Proton-conducting pathways in heme-copper oxidases

Dioxygen reduction releases a large amount of energy (ΔG of −231.8 kJ/mol) [55], which CcO uses to translocate protons across the inner mitochondrial membrane in mammals or plasma membrane in bacteria [70, 130]. Four electrons and four protons (chemical protons) are delivered to the active site in order to convert O₂ to two molecules of H₂O; in addition, four protons are pumped across the membrane [70, 130]. Structural and mutational studies [15] have identified two proton-conducting pathways in subunit I of the *aa*₃ cytochrome *c* oxidases: the D-pathway and the K-pathway, named after conserved aspartate and lysine residues, respectively, located within the channels (Figure 21). The D-channel transfers chemical and pumped protons, whereas the K-channel only transfers chemical protons. Another pathway,
Figure 19. Subunit II amino acid sequence alignment of *R. sphaeroides aa3* cytochrome *c* oxidase and *E. coli bo3* ubiquinol oxidase.
Figure 20. Subunit III amino acid sequence alignment of *R. sphaeroides aa3* cytochrome *c* oxidase and *E. coli bo3* ubiquinol oxidase.
Figure 21. The D-proton pathway and K-proton pathway in *R. sphaeroides aa₃* cytochrome *c* oxidase (PDB 1M56). Residues in the D-channel are shown in yellow. Residues of the K-channel are shown in red. Image created using Pymol.
the H-channel, has also been proposed for the bovine CcO [131]; however, the H-pathway does not appear to be functionally important in the bacterial enzyme [132].

The D-pathway originates with aspartate 132 (amino acid numbering based on Rs CcO) and extends through subunit I to glutamate 286. The protonable residues lining the D-channel coordinate a chain of resolved water molecules and form a hydrogen bonded network capable of conducting H$^+$ from D132 to E286 [133]. Residues in the D-channel include: aspartate 132, asparagine 207, asparagine 139, asparagine 121, serine 142, serine 200, serine 201, and glutamate 286. From E286, H$^+$ is either directed to the active site (which is approximately 10 Å away) or to an unknown proton loading site (proton pump site). Beyond E286, the exit pathway for the pumped protons is currently unknown. The identity of the proton loading site is unclear; however potential candidates include the D-propionate group of heme $a_3$ and a conserved arginine (R481) with which it forms an ion pair, or histidine 334 [134].

The K-pathway transfers chemical protons to the active site. The K-channel, which originates with glutamate 101 in subunit II, lies along the interface of subunits I and II; the pathway includes the conserved lysine 362, serine 299, threonine 359, and terminates with tyrosine 288 in subunit I [135]. The residues lining the K-channel coordinate a water chain which leads to the binuclear center.

D- and K-pathways have also been identified in Ec bo$_3$, analogous to the proton-conducting channels found in other aa$_3$-type oxidases [118, 131, 136, 137]. Both channels are located within subunit I. Protons are pumped across the membrane via the D-channel, whereas the K-channel provides the chemical protons for dioxygen.
reduction. The D-channel in Ec bo3 originates with aspartate 135 (E. coli numbering) and proceeds through asparagine 124, threonine 211, asparagine 142, tyrosine 61, threonine 204, serine 145, threonine 201, and threonine 149 [127]. The D-pathway terminates with glutamate 286, which is in close proximity to the binuclear center [127, 138]. The K-channel consists of lysine 362, threonine 359, threonine 352, the hydroxyl group of the hydroxyethyl farnesyl side chain of heme o3, and ends with tyrosine 288 [127].

1.8 Proposed mechanisms of dioxygen reduction in the fully reduced and mixed-valence forms of bovine heart CcO

**Dioxygen reduction in the fully reduced bovine heart CcO**

The reaction of molecular oxygen with cytochrome c oxidase has been studied extensively by a variety of techniques, including resonance Raman, FTIR, and EPR [100, 139-142]. Time resolved optical absorption spectroscopy, in combination with the flow-flash technique developed by Gibson and Greenwood [143, 144], has been especially useful in elucidating the mechanism of dioxygen reduction in CcO [145]. In the flow-flash method, carbon monoxide (CO) is bound to the reduced heme a3 iron. The CO-bound enzyme is then mixed with oxygen saturated buffer in a flow-cell. Subsequent illumination with laser light photodissociates CO from heme a3 in the presence of O2, thereby initiating the O2 reaction. By analyzing the spectral changes over time, a mechanistic scheme may be proposed for dioxygen reduction.
Figure 2 presents a proposed reaction scheme for O$_2$ reduction in the fully reduced bovine CcO [147]. The reaction generates several intermediates which have characteristic spectral properties. Initially, all four redox-active metal centers are reduced: $a_3^{2+}$, Cu$_B^{1+}$, $a_2^{2+}$, and Cu$_A^{1+}$, with CO bound to heme $a_3$; further, the nearby cross-linked tyrosine residue (Tyr 288) is protonated. Photolysis of the fully reduced CO-bound enzyme generates intermediate $R^*$, in which CO is bound to Cu$_B$. $R^*$ subsequently decays with a lifetime of $\sim$1.5 $\mu$s [146], forming $R$, the fully reduced enzyme. O$_2$ binding to heme $a_3$ ($\sim$12 $\mu$s lifetime) results in the formation of compound $A$. The conversion of compound $A$ to intermediate $P_R$ ($\sim$39 $\mu$s lifetime) involves electron transfer from heme $a$ and Cu$_B$, the donation of H$^+$ (possibly from a nearby tyrosine), and cleavage of the O$_2$ double bond. The uptake of an additional proton converts $P_R$ to intermediate $F$ ($\sim$100 $\mu$s lifetime). Intermediate $F$ undergoes an equilibrium between $F_I$ and $F_{II}$, involving electron transfer between Cu$_A$ and heme $a$. With the uptake of another proton and electron transfer to the binuclear center, $F$ decays to $O$ ($\sim$1.5 ms lifetime), the final oxidized intermediate. Classical CO flow-flash studies of the reaction between dioxygen and the bovine heart enzyme in our laboratory have suggested that $P_R$ is a mixture of $A$, $P$, and $F$ [147].

**Dioxygen reduction in the mixed-valence bovine heart CcO**

Under physiological conditions within the cell, cytochrome $c$ oxidase receives reducing equivalents from the mobile electron-carrier, cytochrome $c$. It is plausible that CcO binds O$_2$ prior to the reduction of all four redox-active metal centers [145].
Figure 22. Conventional unidirectional sequential reaction mechanism for dioxygen reduction in the fully reduced bovine heart cytochrome c oxidase. Image from reference [145].
The mixed-valence CcO represents a two-electron reduced form of the enzyme, in which heme $a_3$ and Cu$_B$ are reduced, and heme $a$ and Cu$_A$ are oxidized. The mixed-valence enzyme, which is capable of binding CO, contains the minimum number of electrons (2 electrons) required for dioxygen to bind the heme $a_3$ iron. In the laboratory, the mixed-valence CcO is prepared in the presence of carbon monoxide, with CO bound to the heme $a_3$ Fe$^{2+}$ [148].

The classical CO flow-flash method has been used to investigate the reaction between dioxygen and the mixed-valence bovine heart enzyme, and a reaction mechanism has been proposed (Figure 23) [148]. In the mixed-valence bovine enzyme, photolysis of the heme $a_3$–CO complex triggers a process known as electron backflow. Electron backflow refers to the intramolecular electron transfer from the reduced heme $a_3$ to the oxidized heme $a$. The binding of dioxygen to the active site heme $a_3$ gives rise to compound A ($A_M$). It has been suggested that during splitting of the O$_2$ double bond, a proton and an electron are abstracted from a nearby tyrosine residue, forming the proposed intermediate $P_M$, as well as a neutral tyrosyl radical [100].

In the laboratory, the mixed-valence CcO is able to initiate catalysis, but it fails to reduce dioxygen to water [148]. Instead, catalysis terminates at intermediate $P_M$ due to an insufficient number of electrons to complete catalysis.
Figure 23. Conventional reaction mechanism for dioxygen reduction in the mixed-valence bovine heart cytochrome c oxidase. Image from reference [148].
1.9 Overview and specific aims of the present thesis

This thesis investigates the spectral and kinetic properties of four variants of the \textit{aa}_3\textit{-type cytochrome c oxidase} from \textit{Rhodobacter sphaeroides} (the wild type subunit I-IV fusion enzyme, E286Q mutant, W172Y/F282T double mutant, and G283L mutant) and the \textit{bo}_3 ubiquinol oxidase from \textit{Escherichia coli}. Chapter 2 utilizes multi-wavelength time resolved optical absorption spectroscopy, in combination with the classical CO flow-flash technique, to elucidate the mechanism of dioxygen reduction in the \textit{R. sphaeroides} \textit{aa}_3 wild-type fusion cytochrome \textit{c} oxidase (\textit{Rs aa}_3), in which the C-terminus of subunit I is fused to the N-terminus of subunit IV. The CO flow-flash technique is also employed to investigate dioxygen reduction in the E286Q mutant of \textit{Rs aa}_3, in which glutamate 286 is replaced with glutamine. Glutamate 286 is the terminal amino acid residue of the proton-conducting D-channel in \textit{Rs aa}_3. This residue is coordinated to a chain of resolved water molecules in the D-pathway. Further, E286 serves as a branch point from which protons are directed to the active site or to an unknown proton pump site. Intermediate spectra are extracted from the time-resolved spectral data acquired during \text{O}_2 reduction in the wild-type fusion and E286Q \textit{Rs aa}_3 enzymes, and are compared on a one-to-one basis with bovine model spectra. Reaction mechanisms for dioxygen reduction are proposed for the wild-type fusion and E286Q mutant enzymes.

Chapter 3 investigates the kinetics of NO binding and \text{O}_2 reduction of the wild-type fusion \textit{Rs aa}_3 in the presence and absence of CO. Previous flow-flash studies involving photolabile \text{O}_2- and NO-complexes have suggested that CO
impedes ligand binding by a factor of 10 in the \textit{ba}_3-type cytochrome \textit{c} oxidase from \textit{Thermus thermophilus}. The second-order rate constant for O$_2$ and NO binding to the active site in \textit{T. thermophilus ba}_3 is \(\sim 1 \times 10^9 \text{ M}^{-1} \text{s}^{-1}\) in the \textit{absence} of CO, and \(\sim 1 \times 10^8 \text{ M}^{-1} \text{s}^{-1}\) in the \textit{presence} of CO \cite{149, 150}. In order to determine the impact of CO upon O$_2$ and NO binding in the wild-type fusion \textit{Rs aa}_3 enzyme, time-resolved multi-wavelength flow-flash experiments utilizing photolabile O$_2$- and NO-complexes were performed. Furthermore, the kinetic mechanism of dioxygen reduction in the absence of CO, using a photolabile O$_2$-carrier, is compared to the mechanism of O$_2$ reduction derived from a classical CO flow-flash experiment.

Chapter 4 represents a spectroscopic investigation of two \textit{Rs aa}_3 enzyme variants in which key amino acid residues within a putative ligand channel have been mutated. Crystallographic studies have identified potential ligand channels within subunit I of several heme-copper oxidases \cite{151, 152}. These proposed ligand channels may facilitate and modulate ligand diffusion to the active site. Chapter 4 examines the role of a highly conserved glycine 283 residue within the ligand channel of \textit{Rs aa}_3. G283 is situated between two bulky residues (tryptophan 172 and phenylalanine 282) at the entrance to the binuclear center. The glycine 283 residue, which has the smallest side chain of all the amino acids, was mutated to a relatively larger residue (leucine); the impact of the G283L mutation upon ligand accessibility to the active site was then investigated. The enzyme activity and CO recombination dynamics of the G283L mutant were measured and compared with the behaviour of the wild-type enzyme. Chapter 4 also examines the CO photodissociation and
recombination dynamics of the W172Y/F283 double mutant. In the Rs aa3 enzyme, tryptophan 172 and phenylalanine 283 are bulky amino acids which project into the putative ligand channel, creating a narrowing of the pathway. Tryptophan and phenylalanine in Rs aa3 were mutated to tyrosine and threonine, respectively, in order to mimic the wider ligand channel in Thermus thermophilus ba3.

In Chapter 5, we utilize the classical CO flow-flash method and multi-wavelength detection to investigate the reaction between dioxygen and the mixed-valence Escherichia coli bo3 ubiquinol oxidase (Ec bo3) or the mixed-valence wild-type fusion Rs aa3 cytochrome c oxidase. Ec bo3 and Rs aa3 are both proton pumps which catalyze the four electron reduction of O2 to water. Although both enzymes share similar protein architecture, Ec bo3 lacks the dinuclear CuA site present in Rs aa3 [92, 127]. Also, the mobile electron carrier for Ec bo3 is the membrane-soluble ubiquinol-8, whereas in Rs aa3, the water-soluble cytochrome c is the electron carrier [70, 127]. The spectra of Ec bo3 and Rs aa3 reaction intermediates are extracted from the time-resolved spectral data, and are compared to model bo3 and bovine intermediate spectra.
References


117. Zhu, J., Han, H., Pawate, A. & Gennis, R. B. (2010) Decoupling mutations in the D-channel of the *aa*3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides*
suggest that a continuous hydrogen-bonded chain of waters is essential for proton pumping, *Biochemistry*. 49, 4476-82.


Chapter 2

Investigation of the Reaction Mechanism and Kinetics of

$O_2$ Reduction in the Wild-Type Fusion and E286Q

Mutant of the $aa_3$ Cytochrome $c$ Oxidase from

*Rhodobacter sphaeroides* by Time-Resolved Optical Absorption Spectroscopy
Introduction

The membrane-bound enzyme, cytochrome c oxidase (CcO), serves as the terminal oxidase of the mitochondrial respiratory chain [1, 2]. CcO is responsible for reducing 95% of inhaled O₂ to water, making it essential for aerobic life [3, 4]. CcO couples dioxygen reduction to the concomitant translocation of protons across the inner mitochondrial membrane [5]. The cell uses the resulting electrochemical proton gradient to drive energetically unfavorable reactions, such as the synthesis of ATP.

Yoshikawa et al. published the first crystal structure of bovine heart CcO in 1995, at 2.8 Å resolution [6]. Since that time, the structures of heme-copper oxidases from many bacteria, such as *Rhodobacter sphaeroides*, *Paracoccus denitrificans*, *Thermus thermophilus*, and *Escherichia coli*, have also been resolved by X-ray crystallography [7-10]. In the aa₃-type CcO from *R. sphaeroides* (hereafter referred to as *Rs aa₃*), the "core" subunits (I, II, and III) share high sequence and structural homology with the bovine enzyme. Because bacteria are amenable to genetic manipulation, bacterial oxidases, such as *Rs aa₃*, are often used as a model for the mammalian enzyme [11].

The CO flow-flash method developed by Gibson and Greenwood [12, 13] has been extremely valuable in studying the molecular mechanism of dioxygen reduction in CcO. In this technique, the fully reduced enzyme is treated with CO, which binds to the active site heme as an inhibitor. The CO-bound enzyme is then mixed with O₂-saturated buffer, and a short laser pulse photodissociates the CO, thereby initiating O₂ binding and reduction to water. This technique is possible because the thermal
dissociation of CO in the dark is slow (~0.03 s\(^{-1}\)) in bovine heart cytochrome c oxidase [12]. A slow CO off-rate in the dark prevents CO from dissociating during the mixing of the sample with O\(_2\)-saturated buffer before the laser pulse triggers the reaction. The CO flow-flash technique, in combination with time-resolved optical absorption (TROA) spectroscopy, has been used to investigate the reaction of dioxygen with bovine heart \(aa_3\) CcO [14]. A kinetic analysis of the CO flow-flash data revealed that bovine heart CcO binds O\(_2\) with a second-order rate constant of ~1×10\(^8\) M\(^{-1}\)s\(^{-1}\) [12].

Single wavelength TROA spectroscopy, in combination with the CO flow-flash method, has been used to probe the kinetics of O\(_2\) reduction in \(Rs\ aa_3\) [15]. These experiments involved laser-induced photolysis of CO from heme \(a_3\), followed by binding of molecular oxygen to the active site; the time-course of the O\(_2\) reduction reaction was then monitored at a single wavelength (or a few selected wavelengths). While these studies yielded important information about the rates observed during dioxygen reduction in \(Rs\ aa_3\) [15, 16], the spectral resolution of single wavelength experiments is inherently limited, hampering the accurate determination of intermediate spectra.

Previous studies reported four rates for the reaction between dioxygen and the fully reduced \(Rs\ aa_3\) (Table 1) [16]. These rates were interpreted in terms of the same conventional sequential mechanism proposed for bovine heart CcO (Chapter 1, Figure 22). In this mechanism, photolysis of the heme \(a_3\)-CO complex generates the fully reduced enzyme, \(R\). Dioxygen then binds to the iron of heme \(a_3\), producing a
<table>
<thead>
<tr>
<th>Reaction step</th>
<th>τ values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R \rightarrow A$</td>
<td>8 μs</td>
</tr>
<tr>
<td>$A \rightarrow P$</td>
<td>50 μs</td>
</tr>
<tr>
<td>$P \rightarrow F$</td>
<td>120 μs</td>
</tr>
<tr>
<td>$F \rightarrow O$</td>
<td>1.2 ms</td>
</tr>
</tbody>
</table>

**Table 1.** Reported rate constants of the reaction between $O_2$ and the fully reduced, CO-bound wild-type $Rs$ *aa3*. The $O_2$ concentration after mixing was approximately 1 mM. From Brzezinski and Adelroth (1998) [16].
ferrous heme $a_3$-O$_2$ adduct known as compound A (also referred to as AR$^1$). Next, electron transfer to the binuclear center cleaves the O-O double bond, forming intermediate PR. The uptake of a proton from solution gives rise to an oxoferryl species called intermediate F, which undergoes a redox equilibrium electron transfer between heme a and Cu$_A$. Heme a is oxidized (Fe$^{3+}$) in F$_I$, but is reduced (Fe$^{2+}$) in F$_II$. Uptake of another proton, along with electron transfer to the active site, results in the formation of the fully oxidized enzyme, O.

Single wavelength CO flow-flash studies have suggested that the O$_2$ reduction reaction terminates at intermediate PR in the E286Q Rs aa$_3$ mutant, in which a key glutamate residue in the D channel has been replaced with glutamine (Figure 1) [17]. Because the conversion of PR to F involves proton uptake, the single wavelength results indicated that glutamate 286 may act as a proton donor during this reaction step. E286 may also serve as a proton donor during the conversion of F to O [17, 18].

While the kinetic resolution of single wavelength TROA spectroscopy can be very high, this method monitors the reaction at only a few selected wavelengths, and consequently, the spectral resolution is inherently limited. As an alternative, multi-wavelength detection may be used in combination with the CO flow-flash technique [14, 19]. In this approach, CO photolysis initiates the reaction with O$_2$, and the spectral changes are monitored over a broad span of times and wavelengths encompassing the Soret and visible regions. Global kinetic analysis of the multi-

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$^1$ The subscript R in AR denotes that compound A has been generated from the fully reduced enzyme; in contrast, the subscript M in AM indicates that intermediate A has been generated from the mixed-valence state. Likewise, PR has been generated from the fully-reduced enzyme, and PM from the mixed-valence enzyme.
Figure 1. The amino acid structures of glutamic acid (left) and glutamine (right).

Images prepared using ChemSketch, version 14.01.
wavelength data yields the apparent rates for each reaction step; the intermediate spectra determined based on a proposed kinetic mechanism, may then be used to evaluate the validity of the reaction scheme.

Multi-wavelength CO flow-flash studies have been successfully performed on the fully reduced CO-bound (FRCO) bovine heart (aa3) [14, 19] and *T. thermophilus* (ba3) oxidases [20, 21]. In the reaction between dioxygen and the fully reduced bovine heart enzyme, it was shown that the spectrum of intermediate P_R differed from the model spectrum of bench-made P (made by reacting the bovine enzyme with H_2O_2) or the experimental intermediate spectrum of P_M generated during the reaction of the mixed-valence bovine enzyme with O_2; rather, P_R was better modeled by a combination of the spectra of intermediates A, P, and F [19]. As a result, the conventional unidirectional sequential mechanism for the bovine enzyme was refined to a branched mechanism, in which one branch produced P, and the other, F [22].

The present work utilizes the CO flow-flash technique, in combination with multi-wavelength TROA spectroscopy, to elucidate the kinetic mechanism of O_2 reduction in the wild-type fusion and E286Q *Rs aa3* enzymes in the presence of CO. Global analysis of the wild-type TROA data and mechanistic analysis suggest that intermediate P_R is absent from the reaction scheme of the wild-type fusion *Rs aa3* and that compound A_R is directly converted to O. However, P_R is generated during the reaction between dioxygen and the E286Q enzyme. The results indicate significant differences in the O_2 reduction kinetics of the bovine heart and *Rs aa3* enzymes. These results were published in *Biochemistry* in 2012 [23].
Materials and Methods

All chemicals were of the highest purity reagent grade. The detergent, n-dodecyl-\(\beta\)-D-maltopyranoside (DM), was obtained from Anatrace. Ni-NTA resin was obtained from Qiagen. Standard grade (99.9\%) carbon monoxide was purchased from Praxair. All other chemicals were from Fisher Biosciences and Sigma-Aldrich. The \textit{R. sphaeroides} wild-type fusion strain was generously provided by Professor Shelagh Ferguson-Miller, Michigan State University. The E286Q \textit{R. sphaeroides} strain was generously provided by Professor Robert Gennis, University of Illinois at Urbana-Champaign. Analysis (and interpretation) of the structural differences between the wild-type fusion and E286Q \textit{Rs aa}_3 enzymes was accomplished by William McDonald, a post-doctoral researcher in our laboratory.

The wild-type fusion \textit{Rs aa}_3

The present study utilized a wild-type fusion enzyme in which subunits I and IV of \textit{R. sphaeroides} cytochrome \(c\) oxidase were fused together (Figures 2 and 3). The C-terminus of subunit I was fused end-to-end with the N-terminus of subunit IV in order to create a protein with a more stable, complete subunit composition and stoichiometry. The wild-type fusion enzyme was engineered with a 6-histidine-tag on subunit II [24], and was expressed in a \(\Delta\text{I}\Delta\text{IV}\) strain of \textit{R. sphaeroides}—a strain in which the genes for subunits I and IV have been deleted from the genome (C. Hiser, personal communication, April 21, 2011).
MADAAIHGEHDDRGFFTRWFSTNHKDIGVLYLFTGGGLVGLISVAFTVYM
RMELMAPGVFQFMCAEHLESGLVKGFFQSLWPSAVENCTPNGHLWNVMITG
HGILMMFFVVIPALFFGFGNYFMPHIGAPDFPRMNNLSYWLIVAGTLS
AVASLFAPGGNGQLSGIGWVLYPPLSTSESGYSTDLALFAVHLGASSILGAI
NMITTFLNMRAPGMHTKHVFPLFAWSIFVTAWLILLALPVLAGAITMOLLDRN
FTTFFQPSPGGDVPVLQHILWFFGHPEVYIIVLPAPGIVSVHIAATFAKKPIFGY
LPMVYAMVAIGVLGFVWVWAHMYTALSTTQQSYFMATMVAVAFTGKIF
SWIATMWGGSIELKTMLWALGFELFTVGGVTIVLSQASVDYHYDITYY
VVAHFHYVMSLGAVFGIFAGIYFWIKMSGRQYPEWAGKHLHFWMVFVAN
LTFFPQHFLGRQGMPRYYIDYPEAFATWNVSSLGAFLSFASFLFFLGVFYTL
TRGARVTANNYWNEHADTLEWTLTSSPPEHTFQLPKREDWERAPAHMAET
NKGTGPMADHSPAHPHVGAMSMDITQQEKTFAGFVRMVTWAAVVIVAALI
FLALANA

Figure 2. The amino acid sequence of the wild-type fusion cytochrome c oxidase from *R. sphaeroides*. The full length of subunit I is fused to the full length of subunit IV. Subunit I is shown in black; subunit IV is shown in blue. (Courtesy of Carrie Hiser, Michigan State University).
Figure 3. DNA sequence of the cytochrome c oxidase subunit I-IV fusion construct in *R. sphaeroides*. The plasmid construct carries the subunit I promoter, the full-length subunit I coding region, the full-length subunit IV coding region, and the subunit IV 3’ untranslated region (terminator). Subunit I is shown in black; subunit IV in blue; added *Hin*III sites in red; and coding regions are shown in bold. (Courtesy of Carrie Hiser, Michigan State University).
The wild-type fusion enzyme was engineered to maximize the homogeneity of the purified protein sample. Previous preparations of the native enzyme suffered from heterogeneity issues [25]. In the native enzyme, incomplete processing of the C-terminus of subunit II gives rise to an inhomogeneous mixture of subunit II polypeptides. Furthermore, native enzyme preparations contain two versions of subunit IV (long and short forms) due to the presence of two translation start codons in the gene encoding subunit IV. The wild-type fusion enzyme circumvents these heterogeneity problems. Placement of a 6-histidine tag on the C-terminus of subunit II ensures a more uniform subunit II peptide. Creation of the subunit I-IV fusion construct (and deletion of subunit IV from the *R. sphaeroides* genome) also eliminates the heterogeneity previously associated with the subunit IV peptide.

**The E286Q Rs aa3**

In the E286Q enzyme, a glutamic acid residue at position 286 (*R. sphaeroides* numbering) has been replaced with glutamine (Figure 4). The E286Q Rs aa3 was constructed using a plasmid (pJS3) containing the *ctaD* gene, which encodes subunit I of cytochrome *c* oxidase [26]. The E286Q enzyme was engineered with a 6-histidine-tag on the C-terminus of subunit I [26], and it was expressed in a *R. sphaeroides* strain in which the *ctaD* gene has been eliminated from the genome [27].
Figure 4. Amino acid sequence alignment of subunit I in the wild-type fusion and E286Q Rs aa3 enzymes. The location of the amino acid substitution is shown in red.
Growth of bacteria and enzyme purification

Figure 5 summarizes the procedure for bacterial growth. The wild-type fusion and E286Q strains were grown in Sistrom’s minimal media [28, 29], supplemented with 50 μg/mL spectinomycin, 50 μg/mL streptomycin, 1 μg/mL tetracycline, and 10 μg/mL of vitamins (final concentrations). Bacteria were cultured aerobically in the dark at 30°C with constant shaking (250 rpm) in a New Brunswick G-25 incubator-shaker. Growth of R. sphaeroides involved a multi-step process, starting with a 50 mL culture (pH 7) in a 500 mL baffled glass flask. When the pH reached 8.5 (approximately 24-48 hours), the culture was transferred to five 500 mL baffled flasks, each containing 100 mL media (pH 7). After inoculating the bacteria, O₂ was bubbled into the media for ~1 minute, and the flasks were subsequently placed in the shaker. When the pH reached 8.5 (another 24 to 48 hours), the culture was transferred to 10 2 L baffled flasks, each containing 700 mL media (pH 7). O₂ was bubbled into the media for ~1 minute, and the flasks were subsequently placed in the shaker.

The cells were harvested in the late exponential phase at pH 8.5 - 9.0 by centrifugation at 8000 rpm (4°C for ~16 minutes) using a Sorvall SLA-3000 rotor. The R. sphaeroides cells were collected as a pellet and stored in a falcon tube at -80°C until further use.

In this study, the isolation and purification of R. sphaeroides CcO closely followed the protocol outlined by Mitchell and Gennis (1995) [30], with some modifications. Figure 6 summarizes the protocol for the enzyme isolation and purification. The harvested cells were resuspended in a cell-breaking buffer (50 mM
Inoculate 50 mL Sistrom’s media (supplemented with spectinomycin, streptomycin, tetracycline, and vitamins) using frozen cell stock

Grow aerobically in the dark at 30°C and 250 RPM until pH > 8.5 (up to 48 hours)

Transfer the culture to five flasks, each containing 100 mL Sistrom’s media (with antibiotics and vitamins). Bubble oxygen into the media for 1 minute

Grow aerobically in the dark at 30°C and 250 RPM until pH > 8.5 (up to 48 hours)

Transfer the culture to ten flasks, each containing 700 mL Sistrom’s media (with antibiotics and vitamins). Bubble oxygen into the media for 1 minute

Grow aerobically in the dark at 30°C and 250 RPM until the cells reach late exponential phase (up to 48 hours)

Harvest the cells at 4°C at 8000 RPM for ~16 minutes

Store cells at -80°C until further use

Figure 5. Flow chart for the growth of *R. sphaeroides*. 
Resuspend and homogenize cells in a buffer containing 50 mM KH₂PO₄, and 1 mM EDTA (pH 6.5) in the presence of 10 mM of MgSO₄, DNase, and protease inhibitor.

Break cells open using a laboratory French Press (approx. 20,000 psi).

Centrifugation at 4°C and 12,000 RPM for 80 minutes to pellet unbroken cells. Collect supernatant.

Ultracentrifugation at 4°C and 52,000 RPM for 90 minutes to isolate the bacterial membranes as a pellet.

Membrane solubilization in n-dodecyl-β-D-maltopyranoside

Ultracentrifugation at 4°C and 52,000 RPM for 30 minutes to remove any unsolubilized material

Perform Ni-NTA affinity chromatography (imidazole concentration gradient) to purify the His-tagged oxidase

Concentrate and wash the eluted enzyme.

Perform anion exchange chromatography (KCl salt gradient)

Concentrate and wash the eluted enzyme. Store at -80°C.

**Figure 6.** Isolation and purification of *R. sphaeroides* cytochrome *c* oxidase.
KH₂PO₄, 1 mM EDTA, pH 6.5) in the presence of 10 mM of MgSO₄, DNase, and protease inhibitor cocktail (50 μL per 40 g of cells), and subsequently homogenized using a hand-homogenizer. The homogenized cells were then lysed by passing them through a laboratory French Press two times at ~20,000 psi (lb/in²). The strong cell wall of *R. sphaeroides* necessitated the use of high pressure to achieve cell lysis.

Centrifugation at 12,000 rpm (4°C for 80 minutes) using a Sorvall SLA-3000 rotor pelleted out any unbroken cells and unwanted debris. The supernatant was collected and ultracentrifuged at 4°C and 52,000 rpm for 90 minutes using a Beckman Ti-70 rotor in a Beckman L8-80M ultracentrifuge. This ultracentrifugation step separates out the bacterial plasma membrane as pellet. The pellet was collected and the membranes were resuspended in membrane-solubilizing buffer (10 mM Tris-HCl, 40 mM KCl, pH 8.00). The resuspended membranes were homogenized using a hand-homogenizer and solubilized in ~3% n-dodecyl-β-D-maltopyranoside (DM) detergent by stirring overnight at 4°C. The solution was then ultracentrifuged at 4°C and 52,000 rpm for 30 minutes in order to remove any unsolubilized material. The supernatant, which contains the His-tagged oxidase, was collected and the enzyme was subsequently purified using Ni-NTA affinity resin.

All steps during the Ni-NTA chromatography process were performed at 4°C. First, the Ni-NTA agarose resin was equilibrated in a membrane-solubilizing buffer (10 mM Tris-HCl, 40 mM KCl, pH 8). The crude enzyme extract was stirred with the equilibrated nickel resin for at least one hour at 4°C to ensure sufficient time for the enzyme to bind the resin. The enzyme-resin slurry was poured into a column, and
once the slurry settled, the resin was washed with approximately 10-15 bed volumes of membrane-solubilizing buffer. An imidazole concentration gradient (5 mM, 10 mM, 15 mM, and 20 mM) was applied to the column. First 5 mM imidazole (in membrane-solubilizing buffer, 0.1% DM) was applied until the eluate from the column turned clear. Next, 10 mM, 15 mM, and 20 mM imidazole were similarly applied to the column until the eluate dripped clear. The His-tagged wild-type fusion oxidase was eluted slowly with membrane-solubilizing buffer containing 150 mM imidazole and 0.1% DM. Fractions were collected during the elution process; the green fraction signified the presence of the eluted enzyme. The purified protein was concentrated using a Vivaspin ultrafiltration concentrator (10,000 molecular weight cutoff) and washed three times with protein storage buffer (10 mM KH$_2$PO$_4$, 1 mM EDTA, pH 7.6, and 0.1% DM). The concentrated wild-type fusion and E286Q enzymes were further purified by anion exchange chromatography.

The ion exchange chromatography was performed at 4°C and utilized TSKgel 5PW-DEAE resin. First, the TSKgel 5PW-DEAE resin was washed with ~5 column bed volumes of an equilibration buffer containing 10 mM KH$_2$PO$_4$, 0.1% DM, and 1 mM EDTA (pH 7.6). The enzyme was applied to the equilibrated resin, and a salt gradient (0 M KCl, 0.1 M KCl, 0.15 M KCl, 0.2 M KCl in potassium phosphate equilibration buffer) was subsequently applied to the column. First, 3 column bed volumes of 0 M KCl (in equilibration buffer) were applied to the column. Next, 0.1 M KCl and 0.15 M KCl were similarly applied to the column. The 0.2 M KCl was then applied to the column to elute the enzyme. Fractions were collected during the elution.
process, and the purified protein was concentrated using a Vivaspin ultrafiltration concentrator (10,000 molecular weight cutoff). The enzyme was washed three times with protein storage buffer (10 mM KH₂PO₄, 1 mM EDTA, pH 7.6, and 0.1% DM). The concentrated wild-type fusion and E286Q enzymes were stored at -80°C until further use.

**Preparation of the fully reduced CO-bound enzymes**

The fully reduced CO-bound wild-type fusion and E286Q Rs aa₃ enzymes were prepared in the following manner: the respective oxidized enzyme sample was diluted in 50 mM sodium phosphate buffer containing 0.1% DM (pH 7.4) or 10 mM KH₂PO₄ buffer containing 1 mM EDTA and 0.1% DM (pH 7.4). The enzyme sample was then deoxygenated by several cycles of evacuation on a vacuum line followed by flushing with inert nitrogen gas. After deoxygenation, the following compounds were added under N₂ gas: 3.6 mg/mL glucose, 0.25 mg/mL glucose oxidase, and 0.01 mg/mL catalase (final concentrations) to ensure an anaerobic environment. Next, the reducing agent, sodium ascorbate (final concentration 1 mM), and the redox-mediator, ruthenium hexamine chloride (final concentration 0.5 μM), were added to the enzyme under N₂ gas. The presence of the fully reduced wild-type fusion enzyme was confirmed by its Soret and visible spectra.

The fully reduced CO-bound enzymes (in which CO is bound to heme a₃) were prepared by exposing the respective fully reduced CcO to CO gas for ~30 minutes, with occasional agitation. The presence of the fully reduced CO-bound wild-
type fusion and E286Q enzyme complexes were confirmed spectrally. Ground state spectra (oxidized, reduced, and reduced CO-bound) were recorded on a Hewlett-Packard 8453 UV/Vis spectrophotometer.

**CO flash-photolysis of the fully reduced wild-type fusion and E286Q enzymes**

CO recombination following photolysis of the fully reduced CO-bound wild-type fusion Rs aa3 was investigated using TROA spectroscopy. Figure 7 shows the experimental set-up for acquiring the TROA spectra during a typical CO flash-photolysis experiment. The CO ligand was photolyzed from heme a3 by a 532 nm laser flash (Q-switched DCR-11 Nd:YAG laser, 7 ns duration pulse). TROA spectra were recorded in the 350 – 700 nm region, at logarithmically spaced time points between 100 ns to 500 ms for both the wild-type fusion and E286Q enzymes. The probe beam, a high-power Xenon flash lamp, was passed through the sample and filters and then was focused into a spectrograph. The enzyme sample was placed in a 10 mm x 4 mm quartz cuvette (Starna Cells, Inc.), and a laser pulse was applied along the 10 mm path length, while the probe beam was applied along the 4 mm path length, 90° to the laser photolysis beam. The TROA signals were recorded by an ICCD camera (Andor Technology) both before and after the laser flash, and difference spectra (post- minus pre-photolysis) were calculated. The TROA difference spectra were analyzed by singular value decomposition (SVD) and global exponential fitting using Matlab (Mathworks), which provides the apparent rates (lifetimes) and the
Figure 7. Experimental set-up for measuring TROA spectra during a CO flash-photolysis experiment.
associated spectral changes (b-spectra) (Refer to the Appendix for more detail on the SVD and global exponential fit analysis).

**Conventional CO flow-flash experiment of the reaction between O2 and the fully reduced wild-type fusion or E286Q Rs aa3**

The reaction between the fully reduced wild-type fusion Rs aa3 and O2 in the presence of CO was investigated by the conventional single-laser CO flow-flash technique [12, 13]. Figure 8 shows the experimental set-up for acquiring the TROA spectra. The fully reduced CO-bound (FRCO) enzyme was transferred into one of the drive syringes of the flow-flash system. The O2-saturated phosphate buffer (50 mM sodium phosphate, pH 7.5) was transferred into the other drive syringe. The FRCO enzyme complex was mixed in a 1:1 volume ratio with the O2-saturated buffer. The O2 reduction reaction was initiated by photolysis of the FRCO enzyme with 532 nm laser light. TROA spectra were recorded in the 350 – 700 nm region over a time period from 100 ns to 200 ms after CO photolysis. The TROA difference spectra were analyzed by SVD and global exponential fitting using Matlab (Mathworks). The apparent rates (lifetimes) and associated spectral changes (b-spectra) were determined, and intermediate spectra were calculated based upon a sequential kinetic scheme.
Figure 8. Experimental set-up for measuring TROA spectra during a conventional CO flow-flash experiment of the reaction of the fully reduced \textit{Rs aa}_3 with \textit{O}_2.
Results

*Spectral properties of the wild-type fusion Rs aa3*

Figure 9A shows the ground state optical absorption spectra of the oxidized, fully reduced, and fully reduced CO-bound (FRCO) wild-type fusion Rs aa3. In the oxidized enzyme, both hemes (a and a3) contain ferric iron (Fe3+). Oxidized Rs aa3 exhibits an intense peak in the Soret region at 422 nm and an α-band in the visible region at 602 nm. Upon reduction, the Soret peak shifts to 445 nm, and the α-band shifts to 606 nm. The 445 nm peak exhibits a small shoulder at approximately 422 nm. In the reduced enzyme, both hemes contain ferrous iron (Fe2+). When the reduced enzyme is exposed to CO, the high-spin heme a3 binds CO, forming a low-spin heme a3-CO complex. Formation of the fully reduced CO-bound enzyme results in absorption at ~435 nm and ~590 nm, due to the heme a3-CO complex. The shoulder at ~444 nm is due to the reduced low-spin heme a. CuA has minor absorbance in the 520 nm region [31], while the absorbance contribution of CuB is unknown.

Figure 9B shows the reduced-minus-oxidized and the reduced-minus-FRCO difference spectra in blue and green, respectively, for the wild-type fusion Rs aa3. The reduced-minus-oxidized difference spectrum shows absorption maxima at ~446 nm and ~607 nm, as well as a negative peak at ~415 nm. The reduced-minus-oxidized extinction coefficients of cytochrome a (Δε = 20.5 mM⁻¹cm⁻¹ at 605 nm) and cytochrome a3 (Δε = 4.8 mM⁻¹cm⁻¹ at 603 nm) in bovine heart CcO suggest that heme a is the primary contributor in the α-band region [32]. However, heme a3 is the
Figure 9. The static optical absorption spectra of the wild-type fusion Rs aa3. (A) The static spectra of the oxidized enzyme (blue), the reduced enzyme (green), and the reduced CO-bound enzyme (red). (B) The difference spectra of the reduced-minus-oxidized enzyme (blue) and the reduced-minus-reduced CO enzyme (green).
primary absorber in the Soret region ($\Delta \varepsilon = 112 \text{ mM}^{-1}\text{cm}^{-1}$ at 444 nm for cytochrome $a_3$ and $\Delta \varepsilon = 57 \text{ mM}^{-1}\text{cm}^{-1}$ at 445 nm cytochrome $a$) [32].

**Spectral properties of the E286Q Rs $aa_3$**

Figure 10A shows the ground state optical absorption spectra of the oxidized, reduced, and reduced CO-bound E286Q Rs $aa_3$. The oxidized spectrum shows a Soret peak at 426 nm and an $\alpha$-band at 599 nm. The reduced E286Q mutant exhibits an intense peak at 444 nm, with a shoulder at ~422 nm. The reduced spectrum also contains an $\alpha$-band at 605 nm. The FRCO spectrum exhibits a 440 nm peak in the Soret region and a 603 nm peak in the visible region, with a slight shoulder at ~590 nm, which is due to CO binding to heme $a_3$. The spectral properties of the E286Q enzyme are in good agreement with the wild-type fusion Rs $aa_3$.

Figure 10B shows the reduced-minus-oxidized and reduced-minus-FRCO difference spectra in blue and green, respectively, for the E286Q enzyme. The reduced-minus-oxidized difference spectrum exhibits a negative peak at ~416 nm and an intense positive peak at ~446 nm. The reduced-minus-oxidized difference spectrum also contains a positive peak at 605 nm, which is predominantly due to reduced heme $a$.

**CO flash-photolysis and TROA spectroscopy of the FRCO wild-type fusion Rs $aa_3$**

CO, a competitive inhibitor of O$_2$, binds to the active site heme $a_3$, and can be photodissociated from heme $a_3$ by a laser pulse, with high quantum yield of CO
Figure 10. The static optical absorption spectra of the E286Q Rs $aa_3$. (A) The static spectra of the oxidized enzyme (blue), the reduced enzyme (green), and the reduced CO-bound enzyme (red). (B) The difference spectra of the reduced-minus-oxidized enzyme (blue) and the reduced-minus-reduced CO enzyme (green).
release [12]. The photodissociation of CO has been utilized as a tool for examining ligand binding dynamics in heme-copper oxidases [33-35]. In this experiment, we photolyzed the fully reduced wild-type fusion Rs aa3-CO enzyme complex and investigated the kinetics of CO recombination to heme a3 by TROA spectroscopy.

Figure 11 shows the TROA difference spectra (post- minus pre-photolysis) recorded during the CO flash-photolysis experiment of the FRCO Rs aa3. Upon photolysis, the low-spin heme a3-CO complex dissociates, generating high spin heme a3 and free CO. As the photolyzed CO recombines with heme a3—thereby regenerating the a3-CO complex—the TROA difference spectra decay to a flat line.

The TROA difference spectra were analyzed using SVD and global exponential fitting (see Appendix). The apparent lifetimes for a four-exponential fit were 880 ns, 94 μs, 16 ms, and 23 ms; the corresponding b-spectra are shown in blue (b1), green (b2), red (b3), and cyan (b4), respectively, in Figure 12A. The non-zero time-dependent b0 spectrum represents the difference spectrum extrapolated to infinite time. Figure 12B shows the residuals, which represent the absorbance differences between the data and the four-exponential fit.

Kinetic analysis of the FRCO Rs aa3 flash photolysis data indicates that CO recombination to heme a3 occurs with a lifetime of 23 ms (Figure 12A, b4, cyan). The other b-spectra are minor in comparison to the large b-spectrum at 23 ms. This experimental CO recombination rate in the wild-type fusion Rs aa3 is in good agreement with the previously determined rate (20 ms) in the native CcO from R. sphaeroides [35].
Figure 11. TROA difference spectra (post- minus pre-photolysis) recorded following photodissociation of the fully reduced CO-bound wild-type fusion Rs aa3. Spectra were recorded in the Soret and visible regions at 21 delay times, logarithmically spaced between 100 ns – 500 ms. The arrows represent the direction of spectral changes with time. Buffer conditions: 10 mM KH$_2$PO$_4$, 1 mM EDTA, 0.1% DM (pH 7.4); optical path length, 0.4 cm.
Figure 12. The $b$-spectra and residuals resulting from a four-exponential fit of the TROA difference spectra acquired following photolysis of the fully reduced CO-bound wild-type fusion $Rs$ $aa3$. Panel A contains the $b$-spectra resulting from the four-exponential fit: $b_1$ (blue, 880 ns), $b_2$ (green, 94 μs), $b_3$ (red, 16 ms), and $b_4$ (cyan, 23 ms). The non-zero time-dependent $b_0$ spectrum (magenta) represents the difference spectrum extrapolated to infinite time. Panel B shows the residuals, which are the difference between the data and the fit, with delay times increasing from bottom to top.
CO flash-photolysis and TROA spectroscopy of the FRCO E286Q Rs aa

The E286 amino acid residue is close to the active site of CcO in \textit{R. sphaeroides}, with the carbonyl carbon of the glutamate side chain \(\sim11.6\ \text{Å}\) away from the heme \(a_3\) iron. Due to the close proximity of this residue to the binuclear center, it is important to determine whether the E286Q mutation impacts the rate of CO recombination to heme \(a_3\). Figure 13 shows the TROA difference spectra (post-minus pre-photolysis) recorded in the Soret and visible regions following photodissociation of the FRCO E286Q enzyme. SVD and global exponential fitting of the spectral data revealed four apparent lifetimes, along with their corresponding \(b\)-spectra (Figure 14A): \(b_1\) (blue, 627 ns), \(b_2\) (green, 85 \(\mu\)s), \(b_3\) (red, 1 ms), and \(b_4\) (cyan, 32 ms). The non-zero time-dependent \(b_0\) spectrum represents the difference spectrum extrapolated to infinite time. The residuals from the four-exponential fit are shown in Figure 14B.

The first three \(b\)-spectra \((b_1,\ \text{blue, 627 ns},\ b_2,\ \text{green, 85 }\mu\text{s},\ \text{and}\ b_3,\ \text{red, 1 ms})\) are minor in comparison to the large \(b\)-spectrum at 32 ms. The apparent lifetime of 32 ms (Figure 14A, \(b_4\), cyan) is attributed to CO recombination. This lifetime is in good agreement with the 23 ms CO rebinding rate in the wild-type enzyme. Thus, the mutation of glutamic acid (at position 286) to glutamate does not appear to impact the CO recombination rate in the \textit{R. sphaeroides} enzyme.
Figure 13: TROA difference spectra (post- minus pre-photolysis) recorded following photodissociation of the fully reduced CO-bound E286Q Rs aa3. Spectra were recorded between 350 – 760 nm at 21 delay times, logarithmically spaced between 100 ns – 0.5 s. The arrows represent the direction of spectral changes with time. Buffer conditions: 10 mM KH₂PO₄, 1 mM EDTA, 0.1% DM (pH 7.4); optical path length 0.4 cm.
Figure 14. The $b$-spectra and residuals resulting from a four-exponential fit of the TROA difference spectra acquired following photolysis of the fully reduced CO-bound E286Q Rs aa3. Panel A contains the $b$-spectra resulting from the four-exponential fit: $b_1$ (blue, 627 ns), $b_2$ (green, 85 μs), $b_3$ (red, 1 ms), and $b_4$ (cyan, 32 ms). The non-zero time-dependent $b_0$ spectrum (magenta) represents the difference spectrum extrapolated to infinite time. Panel B shows the residuals (the difference between the data and the fit) with delay times increasing from bottom to top.
Conventional CO flow-flash experiment of the reaction of O₂ with the fully reduced wild-type fusion Rs aa₃

O₂ binding and reduction by CcO occurs faster than can be resolved by standard mixing techniques. In bovine heart CcO, dioxygen binds heme a₃ within 12 μs (at approximately 600 mM O₂) [22]; however, mixing occurs on the millisecond time scale [36]. Therefore, in order to investigate the enzyme reaction mechanism, a photolabile inhibitor, such as CO, is used to "trap" the enzyme in the CO-bound state. CO thermally dissociates from heme a₃ of bovine CcO relatively slowly (~0.03 s⁻¹ in the dark), allowing sufficient time to mix the enzyme with O₂-saturated buffer before a laser flash is applied [12, 35]. Upon illumination, CO dissociates from heme a₃, thereby initiating the O₂ reduction reaction. This technique, called the CO flow-flash method, has been utilized to investigate the reaction between dioxygen and several heme-copper oxidases [14, 20, 37]; for further review, see reference [34].

Figure 15 shows the TROA difference spectra (post- minus pre-photolysis) recorded in the Soret and visible regions during the reaction of the fully reduced wild-type fusion Rs aa₃ with O₂ in the presence of CO. The TROA difference spectra were analyzed using SVD and global exponential fitting (see Appendix). Multiple exponentials were required to adequately fit the spectra data: 18 μs, 53 μs, and 1.3 ms. An additional lifetime of 0.6 μs was also observed; however, this early process has been attributed to CO photo-release [38]. Figure 16A shows the b-spectra in blue (b₁), green (b₂), and red (b₃), respectively, associated with O₂ reduction. The non-zero time-dependent b₀ spectrum represents the difference spectrum extrapolated to
Figure 15. TROA difference spectra (post- minus pre-photolysis) recorded during the reaction of dioxygen with the fully reduced wild-type fusion Rs aa3 in the presence of CO (conventional CO flow-flash method). The spectra were recorded at 20 delay times, equally spaced on a logarithmic time scale between 100 ns – 200 ms. The arrows represent the direction of spectral changes with time. Conditions: 50 mM sodium phosphate, 0.1% DM (pH 7.5); optical path length: 0.5 cm. The effective enzyme and O2 concentrations after mixing were ~2.7 μM and ~600 μM, respectively.
Figure 16. The $b$-spectra and residuals resulting from a three-exponential fit of the TROA difference spectra acquired during the reaction of the fully reduced wild-type fusion $Rs$ $aa_3$ with O$_2$ in the presence of CO. Panel A contains the $b$-spectra resulting from the three-exponential fit associated with the O$_2$ reduction: $b_1$ (blue, 18 μs), $b_2$ (green, 53 μs), and $b_3$ (red, 1.3 ms)$^2$. The non-zero time-dependent $b_0$ spectrum (cyan) represents the difference spectrum extrapolated to infinite time. Panel B shows the residuals with delay times increasing from bottom to top.

$^2$ An additional lifetime of 0.6 μs was observed but its $b$-spectrum is not included on the graph because it is due to CO photo-release [38] and not the O$_2$ reaction.
infinite time. The good quality of the multi-exponential fit, as shown by the residuals in Figure 16B, indicates that the fit adequately describes the kinetics of O$_2$ reduction in the wild-type fusion $Rs$ $aa_3$.

The $b$-spectra and apparent rates (lifetimes) provide the foundation upon which a kinetic mechanism may be derived. Once a kinetic scheme has been proposed, intermediate spectra may be calculated from the $b$-spectra and apparent lifetimes. The simplest reaction scheme is a unidirectional sequential mechanism in which the experimental apparent rates are assigned as the microscopic rates, in order from the shortest to the longest lifetime, such as the mechanism represented in Scheme 1. Based upon this unidirectional scheme, intermediate spectra were extracted from the $b$-spectra, as described previously [19, 22]. Figure 17 shows the intermediate spectra for the wild-type fusion $Rs$ $aa_3$, referenced versus the fully reduced CO-bound enzyme.

Historically, the mechanism of dioxygen reduction in the bovine enzyme has been represented in terms of a five-intermediate reaction scheme: $R \rightarrow AR \rightarrow PR \rightarrow F \rightarrow O$ [1, 39, 40]. The wild-type fusion $Rs$ $aa_3$ intermediate spectra (referenced versus the oxidized enzyme) generated during the O$_2$ reduction reaction were compared on a one-to-one basis with bovine intermediate spectra generated during O$_2$ reduction in the bovine heart $aa_3$ enzyme. The spectrum of Intermediate 1 of the wild-type fusion $Rs$ $aa_3$ agrees with the spectrum of the first intermediate of the bovine enzyme ($R$), the reduced form of CcO (Figure 18). Furthermore, the spectrum of Intermediate 2 of the wild-type fusion $Rs$ $aa_3$ is also in good agreement with the
Scheme 1. Proposed unidirectional sequential scheme for the reaction between the fully reduced wild-type fusion \textit{Rs \textit{aa}3} and O$_2$ in the presence of CO.
**Figure 17.** The experimental intermediate spectra for the reaction of the fully-reduced wild-type fusion *Rs aat* with O₂ in the presence of CO. The intermediate spectra are referenced versus the reduced CO-bound enzyme. **Intermediate 1** (blue), **Intermediate 2** (green), **Intermediate 3** (red), and **Intermediate 4** (cyan).
Figure 18. Comparison of the first intermediate spectra (referenced versus the respective oxidized enzyme) generated during the reaction between O₂ and the wild-type fusion Rs aa₃ or bovine heart CcO. The experimental spectrum of the first intermediate of the bovine enzyme, R, shown in red, was determined from a five-intermediate unidirectional sequential scheme. The spectrum of the wild-type fusion Rs aa₃ Intermediate 1, shown in blue, was determined from a four-intermediate unidirectional sequential scheme. The spectrum of Intermediate 1 was referenced versus Intermediate 4, the final (fully oxidized) intermediate in the proposed wild-type fusion Rs aa₃ mechanism.
spectrum of the second intermediate of the bovine enzyme (AR), the heme $a_3$ Fe$^{2+}$-O$_2$ complex (Figure 19). However, the spectrum of the wild-type fusion Rs $aa_3$

**Intermediate 3** (Figure 20, blue curve) disagrees with the spectrum of the third intermediate of the bovine enzyme, the so-called $P_R$ intermediate (Figure 20, green curve). Instead, the wild-type fusion Rs $aa_3$ **Intermediate 3** resembles the fourth bovine intermediate, an oxoferryl species, $F$ (Figure 20, red curve), with heme $a$ and Cu$_A$ ~ 60% reduced and oxidized, respectively. Intermediate $F$ is a mixture of $F_I$ and $F_{II}$, where $F_I$ contains Cu$_A^+$ and oxidized heme $a$ (Fe$^{3+}$), and $F_{II}$ contains Cu$_A^{2+}$ and reduced heme $a$ (Fe$^{2+}$).

In order to confirm the identity of the wild-type fusion Rs $aa_3$ **Intermediate 3**, the spectral contribution of reduced heme $a$ was subtracted from the spectrum of **Intermediate 3**, producing the blue curve in Figure 21; only the visible region is shown in Figure 21 because the spectra of bovine $P$ and $F$ are nearly identical in the Soret region. The resulting spectrum is in excellent agreement with the bench-made $F$ spectrum of the bovine heart enzyme, referenced versus the oxidized enzyme, (Figure 21, red curve); both spectra exhibit the characteristic absorbance maximum at ~580 nm. The mechanism for dioxygen reduction in the wild-type fusion Rs $aa_3$ is shown in Figure 22.
Figure 19. Comparison of the second intermediate spectra (referenced versus the respective oxidized enzyme) generated during the reaction between O$_2$ and the wild-type fusion Rs $aa_3$ or bovine heart CcO. The spectrum of the second intermediate of the bovine heart enzyme, A (or $A_R$), shown in red, was determined from a five-intermediate unidirectional sequential scheme. The spectrum of the wild-type fusion Rs $aa_3$ Intermediate 2, shown in blue, was determined from a four-intermediate unidirectional sequential scheme. The spectrum of Intermediate 2 was referenced versus Intermediate 4, the final (fully oxidized) intermediate in the proposed wild-type fusion Rs $aa_3$ mechanism.
Figure 20. Spectral comparison of intermediates 3 and 4 of the bovine heart enzyme (Pr and F, respectively) with the wild-type fusion Rs aa3 Intermediate 3. The spectra of bovine aa3 Pr and F are shown in green and red, respectively, and are referenced versus the oxidized bovine enzyme. The wild-type fusion Rs aa3 Intermediate 3, shown in blue, was determined from a four-intermediate unidirectional sequential scheme, and was referenced versus Intermediate 4, the final (fully oxidized) intermediate in the proposed wild-type fusion Rs aa3 mechanism.
Figure 21. Spectrum of the wild-type fusion Rs aa3 Intermediate 3 (in blue) following subtraction of the spectral contribution of reduced heme a. The spectrum of the bench-made bovine heart aa3 F is shown in red. Both spectra are referenced versus the respective oxidized enzyme.
Figure 22. Proposed mechanism for the reaction between dioxygen and the fully reduced wild-type fusion $Rs~aa_3$ in the presence of CO. TyrOH is postulated to be the cross-linked tyrosine (Tyr 288) [41]. Image modified from [42].
Conventional CO flow-flash experiment of the reaction of $O_2$ with the fully reduced E286Q mutant of Rs $aa_3$

Figure 23 shows the TROA difference spectra (post- minus pre-photolysis) recorded in the Soret and visible regions during the reaction of fully reduced E286Q $Rs aa_3$ with $O_2$ in the presence of CO. The TROA difference spectra were analyzed using SVD and global exponential fitting (see Appendix). SVD and global exponential fitting of the TROA spectra revealed two lifetimes: 16 $\mu$s and 106 $\mu$s. Figure 24A shows the $b$-spectra: $b_1$ (blue) and $b_2$ (green), respectively. The non-zero time-dependent $b_0$ spectrum represents the difference spectrum extrapolated to infinite time. The residuals are shown in Figure 24B. Scheme 2 represents a simple unidirectional sequential mechanism for the reaction of E286Q $Rs aa_3$ with dioxygen in the presence of CO. Based upon this three-intermediate scheme, intermediate spectra were extracted (Figure 25), referenced versus the fully reduced CO-bound enzyme.

The E286Q $Rs aa_3$ intermediate spectra (referenced versus the oxidized enzyme) generated during $O_2$ reduction were directly compared with the wild-type fusion $Rs aa_3$ intermediate spectra. The spectrum of Intermediate 1 of the E286Q $Rs aa_3$ agrees with the spectrum of the wild-type fusion $Rs aa_3$ R, the fully reduced enzyme (Figure 26). The spectrum of Intermediate 2 of the E286Q $Rs aa_3$ is also in good agreement with the spectrum of the wild-type fusion $Rs aa_3$ compound $A_R$ (Figure 27). However, the spectrum of the E286Q $Rs aa_3$ Intermediate 3 (Figure 28, blue curve) disagrees with third intermediate spectrum (F) of the wild-type fusion.
Figure 23. TROA difference spectra (post- minus-pre-photolysis) recorded during the reaction of dioxygen with the fully reduced E286Q Rs aa3 in the presence of CO. The spectra were recorded at 14 delay times, equally spaced on a logarithmic time scale between 1 μs – 20 ms. The arrows represent the direction of spectral changes with time. Conditions: 50 mM sodium phosphate buffer (pH 7.5) with 0.1% DM; optical path length: 0.5 cm. The enzyme and O₂ concentrations after mixing were ~1.7 μM and ~600 μM, respectively.
Figure 24. The $b$-spectra and residuals resulting from a two-exponential fit of the TROA difference spectra acquired during the reaction of the fully reduced E286Q Rs $aa_3$ with O$_2$ in the presence of CO. Panel A contains the $b$-spectra resulting from the two-exponential fit: $b_1$ (blue, 16 μs), $b_2$ (green, 106 μs). The non-zero time-dependent $b_0$ spectrum (red) represents the difference spectrum extrapolated to infinite time. Panel B shows the residuals with delay times increasing from bottom to top.
Scheme 2. Proposed unidirectional sequential scheme for the reaction between the fully reduced E286Q Rs aa3 and O₂ in the presence of CO.
Figure 25. The experimental intermediate spectra for the reaction of the fully reduced E286Q *Rs* aa₃ with O₂ in the presence of CO. The intermediate spectra are referenced versus the reduced CO-bound enzyme. **Intermediate 1** (blue), and **Intermediate 2** (green), **Intermediate 3** (red).
Figure 26. Comparison of the first intermediate spectra (referenced versus the respective oxidized enzyme) generated during the reaction between O$_2$ and the E286Q *Rs* *aa*$_3$ (blue curve) and the wild-type fusion *Rs* *aa*$_3$ (red curve). The spectrum of the E286Q *Rs* *aa*$_3$ Intermediate 1 was determined from a three-intermediate unidirectional sequential scheme. The spectrum of the wild-type fusion *Rs* *aa*$_3$ Intermediate 1 was determined from a four-intermediate unidirectional sequential scheme.
Figure 27. Comparison of the second intermediate spectra (referenced versus the oxidized enzyme) generated during the reaction between $O_2$ and the E286Q Rs aa3 (blue curve) and the wild-type fusion Rs aa3 (red curve). The spectrum of the E286Q Rs aa3 Intermediate 2 was determined from a three-intermediate unidirectional sequential scheme. The spectrum of the wild-type fusion Rs aa3 Intermediate 2 (compound A) was determined from a four-intermediate unidirectional sequential scheme.
Figure 28. Comparison of the spectrum of the bench-made bovine heart P (red curve), with heme a 70% oxidized and 30% reduced, to the spectrum of the E286Q Rs aa3 Intermediate 3 (blue curve), generated during O₂ reduction in the E286Q Rs aa3 in the presence of CO. The spectrum of the E286Q Rs aa3 Intermediate 3 was determined from a three-intermediate unidirectional sequential scheme (Scheme 2). Both spectra are referenced versus the respective oxidized enzyme.
The E286Q Rs aa3 Intermediate 3 also disagrees with the spectrum of Pr from the bovine heart CcO (Figure 20, green curve), which we previously modeled as a mixture of intermediates A, P, and F [19]. Rather, the spectrum of E286Q Rs aa3 Intermediate 3 is best modeled by the bench-made bovine heart P, with heme a ~70% oxidized and ~30% reduced (Figure 28, red curve). These results suggest that the reaction between dioxygen and the E286Q Rs aa3 terminates at P (Pr); the reaction mechanism is summarized in Figure 29. It should be noted that the formation of Pr during O2 in the E286Q Rs aa3 (in the presence of CO) occurs more slowly than in the bovine heart CcO. In the E286Q Rs aa3, the formation of Pr occurs with a lifetime of 106 μs; however, in the bovine enzyme, Pr forms with a lifetime of ~40 μs [19].

Discussion

The kinetic mechanism of O2 reduction in the wild-type fusion Rs aa3 differs significantly from that of the bovine heart CcO. The major difference is that the formation of the so-called Pr intermediate, which is observable in the bovine mechanism, was not detected in the reaction between O2 and the fully reduced wild-type fusion Rs aa3 (in the presence of CO). SVD and global exponential fitting of the wild-type fusion Rs aa3 TROA spectra revealed that three apparent rates (18 μs, 53 μs, and 1.3 ms) were sufficient to describe the dioxygen reduction kinetics in the wild-type fusion Rs aa3. In the bovine enzyme, two lifetimes are associated with the formation of Pr and F; however, only one lifetime was observed in the wild-type
Figure 29. Proposed mechanism for the reaction between dioxygen and the fully reduced E286Q Rs aa3 in the presence of CO.
fusion *Rs aa₃*. These multi-wavelength TROA results suggest that *Pr* is absent from the four-intermediate reaction mechanism proposed for dioxygen reduction in the wild-type fusion *Rs aa₃*.

**Five-intermediate force-fit of the TROA spectra of the wild-type fusion *Rs aa₃***

Previous single-wavelength TROA studies have suggested that the mechanism of dioxygen reduction in the wild-type *Rs aa₃* proceeds through a scheme involving five intermediates: *R* → *A* → *Pr* → *F* → *O* [15, 16]; the published rates were: 16 μs (when adjusted to 600 μM O₂), 55 μs, 130 μs, and 1.3 ms. To explore whether our TROA data would provide both *Pr* and *F* if they were fitted with an additional exponential, the published rates were used to force-fit the wild-type fusion *Rs aa₃* multi-wavelength TROA spectra, resulting in five intermediates. The spectrum of the wild-type fusion *Rs aa₃ Intermediate 3* from the five-intermediate scheme is nearly identical to the spectrum of *Intermediate 3* from the four-intermediate scheme (Figure 20, blue); the spectral agreement is consistent with the fact that the lifetimes associated with the formation of *Intermediate 3* in the five- and four-intermediate schemes are very similar (55 μs for the five-intermediate scheme, 53 μs for the four-intermediate scheme). As observed for the four-intermediate scheme, when the spectral contribution of reduced heme *a* is subtracted from *Intermediate 3* in the five-intermediate scheme, the resulting spectrum is that of the 580 nm *F* species (Figure 21).
Figure 30 compares the spectra of **Intermediates 3** and **4** resulting from the force-fitted five-intermediate scheme. The spectrum of **Intermediate 4** is slightly smaller in amplitude than **Intermediate 3**. This suggests that **F** has decayed to a small extent to the oxidized enzyme, **O**; however, because the change in amplitude is relatively small, the transition from **F** to **O** is largely incomplete. Thus, a five-intermediate force-fit of the wild-type fusion *Rs aa* TROA data unnecessarily splits the transition from **F** → **O** into two steps.

Although **PR** was not detected during the reaction between **O**₂ and the wild-type fusion *Rs aa*₃, its absence does not necessarily eliminate **PR** from a hypothetical reaction mechanism. It could be that intermediate **PR** may not accumulate to detectable levels due to a fast conversion of **PR** → **F** versus a slower **A** → **PR** transition. The conversion of **A** → **PR** involves breakage of the O=O double bond, with the donation of a proton to the binuclear center [1]; the source of this proton may be tyrosine 244 (bovine numbering), which is covalently cross-linked to a histidine ligand of Cu₅ (Y288 is the analogous residue in the wild-type fusion *Rs aa*₃) [43]. The transition from **PR** → **F** may also involve proton transfer, with glutamate 286 as a potential proton donor in *Rs aa*₃ [17]. If intermediates **PR** and **F** differ in their protonation states, then the detection of **PR** may depend upon the relative rates of electron and H⁺ transfer to the binuclear center (BNC). For example, if electron transfer³ from heme *a* to the BNC is faster than proton transfer, then **PR** would be observed following **A**; this may be the case in the reaction between **O**₂ and the fully

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³ In the present study, the rate of electron transfer specifically refers to the rate of heme *a* oxidation.
Figure 30. Spectral comparison of the wild-type fusion Rs aa3 Intermediate 3 (blue) and Intermediate 4 (red) resulting from a force-fitted five-intermediate O2 reduction mechanism. The wild-type fusion Rs aa3 TROA spectra were force-fitted using previously published rates [15, 16]. The spectra are referenced versus the oxidized enzyme.
reduced bovine heart CcO. However, if a proton arrives at the BNC faster than an
electron, then compound A would decay into F; this may be the case for O₂ reduction
in the fully reduced wild-type fusion Rs aa₃.

**Structural differences between bovine heart CcO and R. sphaeroides aa₃**

Electron transfer between heme a and the binuclear center is ~1.5 times faster
in bovine heart CcO (35 μs) than in the wild-type fusion Rs aa₃ (55 μs). Structural
differences between the K- and D-channels of the bovine and Rs aa₃ oxidases may
give rise to different rates of electron and proton transfer. Differences in the identities
of isopositional amino acid residues, as well as differences in H-bonding, may also
contribute to the observed kinetic behavior of the bovine and *R. sphaeroides* enzymes.

Brzezinski et al. (2008) have reported that electron transfer to the BNC may
be linked with charge compensation originating from within the K proton transfer
pathway [44]. In order to achieve this charge compensation, a highly conserved lysine
362 (*R. sphaeroides* numbering) may swing into a position pointing towards the
binuclear center [45]. When the crystal structures of the bovine heart CcO and wild-
type Rs aa₃ are aligned, as shown in Figure 31, numerous structural differences in the
K-channels are apparent. For example, in the bovine heart CcO, serine 255 and
tryptophan 323 (which H-bond to each other) are displaced by ~0.9 Å and 1.0 Å,
respectively, towards the BNC, relative to the corresponding serine 299 and W366
residues in Rs aa₃. Furthermore, lysine 319 in the bovine enzyme is ~0.7 Å closer to
the BNC than the corresponding K362 in Rs aa₃. In particular, the side chain N of the
Figure 31. Structural differences in the K proton transfer channel between the fully reduced bovine heart CcO (PDB 2EIJ, shown in blue) and the wild-type Rs aa3 (PDB 1M56, shown in red). CuB is shown in orange. The heme a3 iron is shown in gray.
bovine K316 is ~0.4 Å closer to the heme $a_3$ iron than the corresponding K362 in $Rs$ $aa_3$. Because the lysine side chain is closer to the active site in the bovine CcO than in $Rs$ $aa_3$, charge compensation (during electron transfer from heme $a$ to the BNC) may be more effective in the bovine enzyme, hence the faster electron transfer in the bovine heart $aa_3$.

Differences in the identities of isopositional residues in the bovine heart and $Rs$ $aa_3$ enzymes may also lead to differential rates of electron and proton transfer (Figure 32). In the wild-type $Rs$ $aa_3$, residue E286 is the terminal residue of the D-channel, and may serve as a source of protons during internal proton transfer. Serine 197 is near E286 and also H-bonds to a water molecule in the D-channel. Due to its close proximity to E286, S197 may be involved with proton loading of E286. In bovine heart CcO, glutamate 242 is the final residue of the D-channel, and alanine 153 is isopositional with $Rs$ $aa_3$ S197. The presence of alanine, rather than a polar residue, may slow proton loading of E242 in bovine heart CcO.

Hydrogen bonding in the bovine heart CcO also differs from that of the wild-type $Rs$ $aa_3$. The heme $a$ propionate of bovine heart CcO forms a hydrogen bond with tyrosine 54 (Figure 33). This H-bond interaction with Y54 causes the propionate group to rotate away from tyrosine 371 (relative to the corresponding tyrosine 414 in the wild-type $Rs$ $aa_3$), resulting in an angle of ~45° of the propionate with respect to the plane of heme $a$. The rotation of the heme $a$ propionate also displaces water molecules in the bovine crystal structure. These structural differences may alter the electrostatic environments within the bovine and wild-type $Rs$ $aa_3$ enzymes,
Figure 32. Structural alignment of the fully reduced bovine heart CcO (PDB 2EIJ, shown in blue) and the wild-type Rs aa₃ (PDB 1M56, shown in red). CuB is shown in orange. The heme a₃ iron is shown in gray.
Figure 33. Hydrogen bonding differences between the heme $a$ propionate groups of the fully reduced bovine heart CeO (PDB 2EIJ, shown in blue) and the wild-type $Rs$ $aa_3$ (PDB 1M56, shown in red). Cu$_B$ is shown in orange. The heme $a_3$ iron is shown in gray.
potentially giving rise to faster electron transfer from heme \(a\) to the BNC in bovine heart CcO, relative to \(Rs\ aa_3\).

The H-bond interactions of one of the heme \(a\) histidine ligands (Figure 34) also differ between the bovine heart \(aa_3\) and wild-type \(Rs\ aa_3\). In the bovine enzyme, histidine 61 is \(\sim 3.16\ \text{Å}\) away from the carbonyl oxygen of glycine 30, which is within H-bonding distance. However, the carbonyl group of the wild-type \(Rs\ aa_3\) serine 44 (the positional equivalent to the G30 of the bovine enzyme) is \(\sim 4.3\ \text{Å}\) away from histidine 102—too far for hydrogen bonding. Instead, H102 forms a hydrogen bond with the hydroxyl group of S44 in the wild-type \(Rs\ aa_3\). These differences in hydrogen bonding near heme \(a\) may contribute to the different rates of electron transfer between the bovine and wild-type \(Rs\ aa_3\) enzymes.

**Structural differences between the wild-type and E286Q \(Rs\ aa_3\) enzymes**

Electron transfer from heme \(a\) to the binuclear center is slower in the E286Q mutant of \(Rs\ aa_3\) (106 \(\mu\)s) than in the wild-type fusion \(Rs\ aa_3\) (53 \(\mu\)s). Structural differences between the wild-type fusion and E286Q \(Rs\ aa_3\) enzymes may contribute to the different rates of electron transfer. Figure 35 shows that the side chain of Q286 in the mutant adopts a different conformation than the E286 in the wild-type enzyme [10]. In the wild-type \(Rs\ aa_3\), glutamate 286 is H-bonded to the carbonyl of methionine 107. However, in the E286Q mutant, the hydrogen bond to M107 is disrupted [10]. The E286Q mutation also causes \(\sim 1.0\ \text{Å}\) displacements of the M107 and W172 side chains [10]. The E286Q mutation displaces water molecules within
Figure 34. Hydrogen bonding differences between histidine 61 of the fully reduced bovine heart CcO (PDB 2EIJ, shown in blue) and histidine 102 of the fully reduced wild-type Rs aa₃ (PDB 1M56, shown in red) enzymes. Cu₈ is shown in orange. The heme a₃ iron is shown in gray.
Figure 35. Structural alignment of the fully reduced wild-type Rs aa3 (PDB 1M56, shown in green) and the fully reduced E286Q Rs aa3 (PDB 1M57, shown in magenta). CuB is shown in orange. Iron is shown in gray.
the D-channel and also causes small displacements of the heme $a$ and heme $a_3$

propionate groups. Furthermore, the E286Q mutation lengthens the H-bond distance
between serine 44 and histidine 102 by $\sim$0.1 Å (Figure 36) relative to the wild-type $Rs$
aa3. Altogether, these structural changes in E286Q $Rs$ aa3 may cause electron transfer
to be slower in the E286Q mutant than in the wild-type fusion $Rs$ aa3.

**Conclusions**

Multi-wavelength TROA studies of the reaction between $O_2$ and the fully
reduced wild-type fusion $Rs$ aa3 (in the presence of CO) have shown that dioxygen
reduction occurs without detectable formation of intermediate P$_R$. Three exponentials
were sufficient to fit the wild-type fusion $Rs$ aa3 TROA spectra, giving rise to a
proposed four-intermediate reaction scheme: $R \rightarrow A \rightarrow F \rightarrow O$. The detection of P$_R$
during dioxygen reduction in the bovine heart CcO, but not in the wild-type fusion $Rs$
aa3, may be due to structural differences between the two enzymes. Structural
changes in the K-proton pathways of the bovine and wild-type fusion $Rs$ aa3 enzymes
may lead to more effective charge compensation during electron transfer from heme $a$
to the binuclear center in the bovine CcO. Structural differences in the D-channels of
the bovine and $Rs$ aa3 enzymes may also slow down proton loading of the bovine
E242 versus the $Rs$ aa3 E286. Differences in the H-bonding interactions of the
propionate groups, as well as of the histidine ligands, may alter the electrostatic
environments within the enzymes, thereby causing electron transfer in the bovine
enzyme to be faster than in the wild-type fusion $Rs$ aa3.
Figure 36. Hydrogen bonding differences between the heme $a$ histidine ligands of the fully reduced wild-type $Rs$ aa$_3$ (PDB 1M56, shown in green) and the fully reduced E286Q $Rs$ aa$_3$ (PDB 1M57, shown in magenta). Cu$_B$ is shown in orange. Iron is shown in gray.
The reaction between dioxygen and the E286Q Rs aa₃ (in the presence of CO) required two exponentials to adequately fit the TROA spectra, giving rise to a proposed three-intermediate scheme: R → A → Pₓ. Electron transfer between heme a and the BNC is slower in the E286Q mutant than in the wild-type fusion Rs aa₃. Different conformations of the E286 and Q286 side chains, as well as displacement of water molecules within the D-channel of the E286Q mutant, may result in the different kinetic behavior of the enzymes. Changes in the H-bonding interactions of residues Q286 and E286, as well as of the heme a histidine ligands, may also contribute to the different rates of electron transfer in the wild-type fusion and E286Q Rs aa₃ enzymes.
References


Chapter 3

Time-Resolved Optical Absorption Spectroscopic Investigation of the Reactions of NO and O\textsubscript{2} with the \textit{aa\textsubscript{3}} Wild-Type Fusion Cytochrome \textit{c} Oxidase from \textit{Rhodobacter sphaeroides} in the Absence and Presence of CO
Introduction

The reduction of molecular oxygen to water is fundamental for life in the aerobic biosphere [1-3]. Heme-copper oxidases (HCOs) catalyze the dioxygen reduction reaction and are responsible for 90 – 95% of the total O2 consumption in aerobic organisms [1, 2]. HCOs are characterized by the presence of a redox-active binuclear center, which contains a penta-coordinate high-spin heme Fe located in close proximity (less than 5 Å) to a copper atom (denoted as CuB) [4-9]. The high-spin heme iron binds diatomic oxygen (as well as other ligands, such as NO, CO, and CN−), and catalysis subsequently takes place at the binuclear center [10].

The HCOs are a diverse superfamily of enzymes that can be divided into three main classes—A, B, and C—based upon genomic and structural evidence [3, 11]; additional families (D, E, F, G, and H) have been suggested elsewhere [12]. The aa3-type cytochrome c oxidases, including bovine heart aa3 and Rhodobacter sphaeroides aa3 (Rs aa3), belong to the A-family. Members of the A-family exhibit low oxygen affinity and are predominantly expressed under aerobic conditions [13]. The ba3-type oxidases from the B-family, such as Thermus thermophilus ba3 (Tt ba3), as well as the cbb3-type oxidases from the C-family, such as R. sphaeroides cbb3, have higher oxygen affinities and are expressed under microaerobic conditions [13]. Phylogenetic analyses of DNA and protein sequences suggest that all heme-copper oxidases are evolutionarily related [3, 11, 12]; the A-family appeared first, while the B- and C-families evolved later.
Although the active site is buried within the interior of the protein, the rate constant for O$_2$ binding in the presence of CO is $\sim 1 \times 10^8$ M$^{-1}$s$^{-1}$ in the bovine heart $aa3$ [14], a value which approaches the diffusion controlled limit ($\sim 10^9$ M$^{-1}$s$^{-1}$) [15]. Classical CO flow-flash studies, in which the reaction with O$_2$ is initiated by photolyzing the reduced CO-bound enzyme in the presence of O$_2$, have also determined that O$_2$ binds to the heme $a_3$ iron with a rate constant of $\sim 1 \times 10^8$ M$^{-1}$s$^{-1}$ in $Rs$ $aa3$ [16], consistent with the rates of O$_2$ and NO binding in the bovine enzyme.

Time-resolved infrared studies have indicated that CO binds to Cu$_B$ following photolysis of the fully reduced CO-bound bovine $aa3$, $Tt$ $ba$, $Escherichia$ $coli$ $ba$, and $Rs$ $aa3$, forming a transient Cu$_B^+$–CO complex (see ref. [10] for details). In $Tt$ $ba3$, which is a distant relative of the A-family and is predominantly expressed under microaerobic conditions [13], the Cu$_B^+$–CO complex decays with a lifetime of $\sim 20$ ms (34.5 s$^{-1}$), which is much slower than O$_2$ binding in the bovine heart $aa3$ ($\sim 10$ μs at 1 mM O$_2$). These findings suggested the possibility that the photodissociated CO might compromise CO flow-flash experiments in $Tt$ $ba3$. Indeed, recent time-resolved optical absorption studies in our laboratory have shown that the presence of CO impedes O$_2$ and NO binding in $Tt$ $ba3$ [17, 18]. In the absence of CO, the rate constant of O$_2$ and NO binding in $Tt$ $ba3$ was $\sim 1 \times 10^9$ M$^{-1}$s$^{-1}$. However, in the presence of CO, the rate was approximately 10 times smaller ($\sim 1 \times 10^8$ M$^{-1}$s$^{-1}$) [17]. These results indicated that the photodissociated CO impedes access of O$_2$ and NO to the active site in $Tt$ $ba3$. Whether this is also the case in the $aa3$ oxidases is unknown.
In this study, we investigated the reaction kinetics of the fully reduced wild-type fusion Rs aa₃ with photoproduced NO in the absence and presence of CO using a photolabile NO complex. In addition, a photolabile O₂-carrier was used to investigate O₂ binding in the absence of CO in the fully-reduced wild-type fusion Rs aa₃, and these results were compared to O₂ binding and reduction in a conventional CO flow-flash experiment. Although the presence of CO slows down ligand binding in Tt ba₃ by a factor of 10, our results show that CO does not impede ligand access to the active site in the wild-type fusion Rs aa₃. Structural differences that may contribute to the differential effects of CO upon ligand binding in the Rs aa₃ and Tt ba₃ enzymes are discussed.

Materials and Methods

The *Rhodobacter sphaeroides* wild-type fusion strain was generously provided by Professor Shelagh Ferguson-Miller, Michigan State University. The *R. sphaeroides* wild-type fusion cytochrome c oxidase, in which the C-terminus of subunit I is fused to the N-terminus of subunit IV, was previously described in the Materials and Methods section of Chapter 1. Protocols pertaining to bacterial growth, as well as enzyme isolation and purification, may be found in Chapter 1.

All chemicals used were of the highest purity reagent grade. The detergent, n-dodecyl-β-D-maltopyranoside (DM), was obtained from Anatrace. Ni-NTA resin was obtained from Qiagen. Standard grade (99.9%) carbon monoxide was purchased from Praxair. The photolabile NO-complex, potassium pentachloronitrosylruthenate(II)
(Ru 25.8%) (PPNR), was obtained from Alfa Aesar. All other chemicals were from Fisher Biosciences and Sigma-Aldrich.

**Synthesis of (μ-peroxo)(μ-hydroxo)bis[bis(bipyridyl) cobalt(III)] nitrate**

The photolabile O₂-complex, (μ-peroxo)(μ-hydroxo)bis[bis(bipyridyl) cobalt(III)] nitrate (HPBC), shown in Figure 1, was prepared according to a previously described protocol [19]. All synthetic steps were performed under red light and in a 37°C bath. The following compounds were mixed together: a solution of 0.0384 moles of 2,2’-dipyridyl in 50 mL ethanol, a solution of 0.01921 moles of cobalt (II) nitrate hexahydrate in 50 mL of ethanol, and 0.096 moles of solid sodium hydroxide. While gently shaking the mixture at ~50 rpm, O₂ gas was bubbled into the mixture for 60 seconds, at which point the color of the solution turned brown. The sample was stirred at ~100 rpm, ~50 rpm, and ~25 rpm for 5 minutes at each speed. After overnight storage in the dark at 30°C, the sample was filtered and washed 3 times with ethanol. The sample was dried in a desiccator under vacuum for 3-7 days, yielding the final O₂-complex. The HPBC complex was wrapped in foil and stored in the dark at -20°C.

**Preparation of the photolabile O₂- and NO-complexes**

For the flow-flash experiments involving photoproduced O₂, a solution of the O₂-complex was prepared by dissolving the solid HPBC complex in a buffer containing 10 mM KH₂PO₄, 1 mM EDTA, and 0.1% DM (pH 7.4). The HPBC
Figure 1. Structure of the $(\mu$-peroxo)$(\mu$-hydroxo)bis[bis(bipyridyl) cobalt(III)] nitrate complex (HPBC). Chemical structure (left) and ball-and-stick structure (right). Image prepared using ChemSketch, version 14.01.
solution was made and used on the same day as the flow-flash experiment was performed. The solid HPBC complex was dissolved in sodium phosphate buffer. The HPBC solution was prepared at room temperature under dimmed room lighting or under red light. The solution was deoxygenated by several cycles of evacuation on a vacuum line, followed by flushing with inert nitrogen gas. After deoxygenation, the following compounds were added under N₂ gas: 3.5 mg/mL glucose, 0.25 mg/mL glucose oxidase, and 0.01 mg/mL catalase (final concentrations). Glucose, glucose oxidase, and catalase were added in order to ensure an anaerobic environment. The HPBC solution was kept in the dark at room temperature until the flow-flash experiment was ready to be performed.

For the photolabile NO-complex experiments, a solution of the commercially available potassium pentachloronitrosylruthenate(II) (PPNR) was prepared in a buffer containing 10 mM KH₂PO₄, 1 mM EDTA, 0.1% DM (pH 7.4). The PPNR was prepared and treated in the same manner as the HPBC solution. The structure of PPNR is shown in Figure 2.

**Preparation of the fully reduced wild-type fusion Rs aa₃ enzyme in the absence and presence of CO**

The wild-type fusion Rs aa₃ was diluted in 50 mM sodium phosphate buffer supplemented with 0.1% DM (pH 7.4). The enzyme sample was deoxygenated by several cycles of evacuation on a vacuum line, followed by flushing with inert nitrogen gas. The glucose, glucose oxidase, and catalase mixture was added to
Figure 2. Structure of the potassium pentachloronitrosylruthenate(II) complex (PPNR). Chemical structure (left) and ball-and-stick structure (right). Image prepared using ChemSketch, version 14.01.
remove any residual $O_2$. The reducing agent, sodium ascorbate (final concentration 1 mM), and the redox mediator, ruthenium hexamine chloride (final concentration 0.5 μM), were then added to the enzyme under $N_2$ gas. The presence of the fully reduced wild-type fusion $Rs$ $aa_3$ was confirmed by its Soret and visible spectra.

For the flow-flash experiments in the presence of CO, the fully reduced CO-bound $Rs$ $aa_3$ enzyme was prepared by exposing the fully reduced wild-type fusion $Rs$ $aa_3$ to CO gas for approximately 30 minutes, with occasional agitation. The presence of the fully reduced CO-bound $Rs$ $aa_3$ was confirmed by its optical absorption spectra. Ground state spectra (oxidized, reduced, and reduced CO-bound) were recorded on a Hewlett-Packard 8453 UV/Vis spectrophotometer.

**Single laser flow-flash experiments of the reaction between the fully reduced wild-type fusion $Rs$ $aa_3$ and photoproduced $O_2$ or NO in the absence of CO**

Reactions between the fully reduced wild-type fusion $Rs$ $aa_3$ and photoproduced $O_2$ or NO in the absence of CO were investigated using time-resolved optical absorption (TROA) spectroscopy. Figure 3 shows the experimental set-up for measuring the TROA spectra during a single laser flow-flash experiment. The deoxygenated fully reduced wild-type fusion $Rs$ $aa_3$ was carefully transferred into one of the drive syringes of the flow-flash system. Buffer containing the deoxygenated photolabile $O_2$- or NO-complex (PPNR) was transferred into the other drive syringe. The enzyme was mixed in a 1:1 volume ratio with the $O_2$- or NO-carrier in a micro-cuvette flow-cell. Photolysis of HPBC or PPNR was achieved with a 355 nm laser
Figure 3. Experimental set-up for measuring TROA spectra during the reaction between the fully reduced $Rs\ aa_3$ and photoproduced $O_2$ or NO in the absence of CO (single laser flow-flash experiment).
flash (Q-switched DCR-11 Nd:YAG laser, 7 ns duration pulse, 3rd harmonic). TROA spectra were recorded in the 350 – 700 nm region over a time period from 5 μs to 500 ms for the reaction with O₂ and 5 μs to 5 ms for the reaction with NO. The probe beam, a high-power Xenon flash lamp at 90° to the laser photolysis flash, was passed through the sample and filters and then was focused into a spectrograph. The TROA signals were recorded by an ICCD camera (Andor Technology) both before and after the laser flash, and difference spectra (post- minus pre-photolysis) were calculated.

In a separate experiment, HPBC or PPNR was mixed in a 1:1 volume ratio with water, and TROA spectra were recorded in the 350 – 700 nm region over a time period from 5 μs to 500 ms (for HPBC) and 5 μs to 5 ms (for PPNR). This experiment was done to determine the spectral characteristics associated with HPBC or PPNR [17, 18]. Subsequently, the spectral contribution from the photolabile carrier was subtracted from the spectra recorded for the reaction of the fully reduced wild-type fusion Rs aa₃ with the photoproduced O₂ or NO. The concentrations of the HPBC and PPNR were determined as described previously [17, 18].

The TROA difference spectra recorded during the reaction of the fully reduced wild-type fusion Rs aa₃ with photoproduced O₂ or NO were analyzed by SVD and global exponential fitting using Matlab (Mathworks). The apparent rates (lifetimes) and associated spectral changes (b-spectra) were determined, and intermediate spectra were calculated based upon a sequential kinetic scheme.
Double laser flow-flash experiment of the reaction between the fully reduced wild-type fusion Rs aa₃ and photoproduced NO in the presence of CO

The reaction between the fully reduced wild-type fusion Rs aa₃ and photoproduced NO in the presence of CO was investigated using a double laser flow-flash technique. Figure 4 shows the experimental set-up. The deoxygenated FRCO enzyme complex was carefully transferred into one of the drive syringes of the flow-flash system. A sodium phosphate buffer containing deoxygenated PPNR was transferred into the other drive syringe. The FRCO enzyme complex was mixed in a 1:1 volume ratio with the NO-complex solution. In the double laser experiment, the reaction was initiated by the simultaneous photolysis of the FRCO Rs aa₃ enzyme and the PPNR complex. A 355 nm laser pulse photolyzed the NO complex, whereas a 532 nm laser pulse photolyzed the FRCO enzyme. Because the photolabile carrier absorbs strongly in the 300 – 400 nm region, the 532 nm laser pulse was necessary for efficient photolysis of CO from heme α₃. TROA spectra were recorded in the 350 – 700 nm region over a time period from 10 μs to 2 ms after photolysis. The TROA difference spectra were analyzed by SVD and global exponential fitting using Matlab (Mathworks). The apparent rates (lifetimes) and associated spectral changes (b-spectra) were determined, and intermediate spectra were calculated based upon a sequential kinetic scheme.
Figure 4. Experimental set-up for measuring TROA spectra during the reaction between the fully reduced $Rs$ $aa_3$ and photoproduced $O_2$ or NO in the presence of CO (double laser flow-flash experiment).
**Conventional CO flow-flash experiment of the reaction between O₂ and the fully reduced wild-type fusion Rs aa₃**

The reaction between the fully reduced wild-type fusion Rs aa₃ and O₂ in the presence of CO was investigated by the conventional single-laser CO flow-flash technique [14, 20], as previously described in the Materials and Methods section in Chapter 2.

**Results**

**TROA measurements of the reaction of the fully reduced wild-type fusion Rs aa₃ with photoproduced NO in the absence of CO: A single laser flow-flash study using a photolabile NO carrier**

Nitric oxide (NO) is a physiologically important ligand in the bacterium R. sphaeroides due to the presence of NO reductase within the bacterial plasma membrane [21]. Furthermore, NO may play a role in regulating respiration by reversibly inhibiting the mitochondrial cytochrome c oxidase [22]. Because NO binding is a single-step process, flow-flash experiments involving NO offer a straightforward way to investigate the ligand binding dynamics of CcO without the complexity of subsequent electron transfer processes, as is the case in experiments involving CcO and dioxygen.

The NO binding dynamics of Rs aa₃ in the absence of CO were investigated by TROA spectroscopy using the photolabile NO-complex PPNR. In this single laser
flow-flash experiment, a 355 nm laser pulse induces photodissociation of NO from the PPNR complex, which initiates the NO binding reaction.

Figure 5 shows the TROA difference spectra (post- minus pre-photolysis) recorded in the Soret and visible regions for the reaction of the fully reduced wild-type fusion \textit{Rs aa3} with photogenerated NO in the absence of CO (before and after subtracting the spectral contribution of the photolabile NO complex).

The first difference spectrum at 5 μs is essentially flat because this spectrum represents the reduced-minus-reduced enzyme, namely, before NO binds to heme \textit{a3}. As the delay times increase, the spectral amplitudes increase as NO binds to the reduced heme \textit{a3} iron. The experimental concentration of the photoproduced NO was determined by obtaining transient difference spectra (post- minus pre-photolysis) of the PPNR complex alone (in a separate experiment) and comparing them to NO photolysis standardization curves at each delay time [18].

The TROA difference spectra were analyzed using SVD and global exponential fitting (see Appendix). The apparent lifetime for a one-exponential fit was 100 μs, with the corresponding \textit{b}-spectrum shown in Figure 6A (\textit{b1}, blue). The non-zero time-dependent \textit{b0} spectrum represents the difference spectrum extrapolated to infinite time. The \textit{b}-spectra may be regarded as the spectral differences between individual intermediates if the reaction mechanism follows a unidirectional sequential mechanism (which is true for the single-step NO binding process) and if the individual processes are well separated in time. The \textit{b1} spectrum represents the spectral change between the first intermediate (the reduced enzyme) and the final
Figure 5. TROA difference spectra (post- minus pre-photolysis) recorded during the reaction of the fully reduced wild-type fusion Rs aa3 with photoproduced NO in the absence of CO. TROA difference spectra before (A) and after (B) subtraction of the spectral contribution from the photolabile NO carrier. The spectra were recorded at ten delay times, equally spaced on a logarithmic time scale between 5 μs – 5 ms. The arrows represent the direction of spectral changes with time. Buffer conditions: 50 mM sodium phosphate, 0.1% DM (pH 7.4); optical path length: 0.5 cm. After mixing, the effective enzyme concentration was ~2.2 μM, and the effective NO concentration was ~104 μM.
Figure 6. The $b$-spectra (Panel A) and residuals (Panel B) resulting from a one-exponential fit of the TROA difference spectra recorded during the reaction of the fully reduced wild-type fusion $Rs$ $aa_3$ with photoproduced NO in the absence of CO.
intermediate (the NO-bound enzyme), while $b_0$ represents the difference between the final intermediate (the NO-bound enzyme) and the original reduced enzyme. Thus, $b_1$ and $b_0$ are mirror images of each other. Figure 6B shows the residuals, which represent the absorbance differences between the data and the one-exponential fit.

Based upon the spectral changes represented by the $b$-spectra, the single apparent lifetime of 100 μs is assigned to the binding of NO to heme $a_3^{2+}$. Figure 7 shows the intermediate spectra (referenced versus the reduced enzyme) generated during the reaction of the fully reduced wild-type fusion $Rs$ $aa_3$ with photoproduced NO. Because NO binding is a single step process, the first intermediate spectrum (Figure 7, blue) represents the photolyzed reduced enzyme (referenced versus the original reduced enzyme), and the second intermediate spectrum (Figure 7, green) represents the NO-bound enzyme (referenced versus the original reduced enzyme). The effective concentration of photoproduced NO was ~104 μM, which together with the 100 μs lifetime, resulted in a second-order rate constant of approximately $1 \times 10^8$ M$^{-1}$s$^{-1}$ for NO binding in $Rs$ $aa_3$ in the absence of CO.

**TROA measurements of the reaction of the fully reduced wild-type fusion $Rs$ $aa_3$ with photoproduced NO in the presence of CO: A double laser flow-flash study using a photolabile NO carrier**

In $Tt$ $ba_3$, the presence of CO impedes O$_2$ and NO binding by a factor of 10 [17, 18]. In order to determine if CO hinders access to the active site in the fully reduced wild-type fusion $Rs$ $aa_3$, NO binding in the presence of CO was investigated
Figure 7. The experimental intermediate spectra for the reaction of the fully reduced wild-type fusion *Rs* *aa*$_3$ with photoproduced NO in the absence of CO. The intermediate spectra are referenced versus the reduced enzyme. **Intermediate 1** is shown in blue and **Intermediate 2** is shown in green.
by TROA spectroscopy using the photolabile NO-complex, PPNR. In this double laser flow-flash experiment, the reaction was initiated by the simultaneous photolysis of NO and CO; a 355 nm laser pulse induced the photorelease of NO from the PPNR complex, and a 532 nm laser pulse photodissociated the enzyme-CO complex.

Figure 8 shows the TROA difference spectra (post- minus pre-photolysis) for the reaction of the fully reduced wild-type fusion Rs aa3 with photogenerated NO in the presence of CO (before and after subtracting the spectral contribution of the photolabile complex). Spectra were recorded in the Soret and visible regions between 10 μs – 2 ms after photolysis.

The TROA difference spectra were analyzed using SVD and global exponential fitting (see Appendix). The apparent lifetime for a one-exponential fit was 81 μs, with the corresponding b-spectrum shown in Figure 9A (b1, blue). The non-zero time-dependent b0 spectrum is shown in green. Figure 9B shows the residuals associated with each time delay. Figure 10 shows the spectra of the intermediates generated during the reaction of the fully reduced wild-type fusion Rs aa3 with photogenerated NO in the presence of CO.

Based upon the spectral changes represented by the b-spectra, the single lifetime of 81 μs is assigned to the binding of NO to heme a32+. The effective concentration of photoproduced NO was determined to be 140 μM, which, taken together with a lifetime of 81 μs, gives rise to a second-order rate constant of ~ 9×10⁷ M⁻¹s⁻¹ for NO binding in the presence of CO. This value is the same, within experimental error, as determined for NO binding in Rs aa3 in the absence of CO.
Figure 8. TROA difference spectra (post- minus pre-photolysis) recorded during the reaction of the fully reduced wild-type fusion Rs aa3 with photoproduced NO in the presence of CO. TROA difference spectra before (A) and after (B) subtraction of the spectral contribution from the photolabile NO-complex. The spectra were recorded at eight delay times, equally spaced on a logarithmic time scale between 10 μs – 2 ms. The arrows represent the direction of spectral changes with time. Buffer conditions: 50 mM sodium phosphate, 0.1% DM (pH 7.4); optical path length: 0.5 cm. After mixing, the effective enzyme concentration was 3.4 μM, and the effective NO concentration was 140 μM.
Figure 9. The $b$-spectra (Panel A) and residuals (Panel B) resulting from a one-exponential-fit of the TROA difference spectra recorded during the reaction of the fully reduced wild-type fusion $Rs$ $aa3$ with photoproduced NO in the presence of CO.
Figure 10. The experimental intermediate spectra generated during the reaction of the fully reduced wild-type fusion *Rs aas* with photoproduced NO in the presence of CO. The intermediate spectra are referenced versus the original reduced CO-bound enzyme. **Intermediate 1** is shown in blue and **Intermediate 2** is shown in green.
Hence, the binding of NO to the active site in the wild-type fusion Rs aa3 is unaffected by CO. Furthermore, the second-order rate constant of NO binding in Rs aa3 in either the presence or absence of CO closely matches the NO binding rate in bovine heart CcO (1×10^8 M⁻¹s⁻¹) [18].

**TROA measurements of the reaction of the fully reduced wild-type fusion Rs aa3 with photoproduced O_2 in the absence of CO: A single laser flow-flash study using a photolabile O_2-complex**

To determine if the presence of CO impedes dioxygen binding in Rs aa3, we investigated the reaction of the fully reduced wild-type fusion Rs aa3 with photoproduced O_2 in the absence of CO using the photolabile O_2-complex (μ-peroxo)(μ-hydroxo)bis[bis(bipyridyl) cobalt(III)] nitrate (HPBC). In this single laser flow-flash experiment, a 355 nm laser pulse induced the photorelease of O_2 from the HPBC complex, initiating the reaction.

Figure 11 shows the TROA difference spectra (post- minus pre-photolysis) recorded in the Soret and visible regions for the reaction of the fully reduced wild-type fusion Rs aa3 with photoproduced O_2 in the absence of CO (before and after subtracting the spectral contribution of the photolabile complex). The underlying trough spanning the 350 – 550 nm region in Figure 11A is due to the photoprodut of the HPBC complex upon O_2 release. Figure 11B shows the spectral changes of the enzyme after subtracting this spectral contribution of the HPBC photoprodut. The
Figure 11. TROA difference spectra (post- minus-pre-photolysis) recorded during the reaction of the fully reduced wild-type fusion Rs aa3 with photogenerated O2 in the absence of CO. TROA difference spectra before (A) and after (B) subtraction of the spectral contribution from the photolabile O2-complex. The spectra were recorded at 16 delay times, equally spaced on a logarithmic time scale between 5 μs – 500 ms. The arrows represent the direction of spectral changes with time. Buffer conditions: 50 mM sodium phosphate, 0.1% DM (pH 7.4); optical path length: 0.5 cm. After mixing, the effective enzyme and photoproduced O2 concentrations were ~1.8 μM and ~40 μM, respectively.
effective O₂ concentration was found using an extinction coefficient of ε₃₉₅ = 4400 M⁻¹cm⁻¹ for the HPBC difference spectra [17, 18].

The TROA difference spectra were analyzed using SVD and global exponential fitting (see Appendix). The apparent lifetimes for a two-exponential fit were 330 μs and 2 ms, with the corresponding b-spectra shown in blue (b₁) and green (b₂), respectively, in Figure 12A. The non-zero time-dependent b₀ spectrum is shown in red. The residuals are shown in Figure 12B.

Scheme 1 represents a three-intermediate unidirectional sequential mechanism in which the intermediates of the reaction between the photoproduced O₂ and the fully reduced wild-type fusion Rs aa₃ (in the presence of CO). Based upon this unidirectional scheme, intermediate spectra were extracted from the b-spectra. Figure 13 shows the extracted intermediate spectra for the wild-type fusion Rs aa₃, referenced versus the fully reduced CO-bound enzyme.

While a four-intermediate scheme (R → A → F → O) describes the O₂ reduction kinetics of the wild-type fusion Rs aa₃ during a conventional CO flow-flash experiment (Chapter 2), the reaction of the fully reduced enzyme with photoproduced O₂ in the absence of CO is adequately represented by three intermediates. This is because of the limited concentration of the photoproduced O₂, which precludes the observation of compound A during the reaction of the enzyme with photogenerated O₂. Figure 14 shows the spectrum of Rs aa₃ Intermediate 1 in the absence of CO (green curve), which is in good agreement with the spectrum of Rs aa₃ R in the presence of CO (blue curve). The spectrum of Rs aa₃ Intermediate 2 in the absence
**Figure 12.** The $b$-spectra (Panel A) and residuals (Panel B) resulting from a two-exponential fit of the TROA difference spectra recorded during the reaction of the fully-reduced wild-type fusion $Rs \, aa_3$ with photoproduced $O_2$ in the absence of CO.
Scheme 1. Proposed unidirectional sequential scheme for the reaction between the fully reduced wild-type fusion \textit{Rs aa}_3 and photoproduced O\textsubscript{2} in the \textit{absence} of CO.
**Figure 13.** The experimental intermediate spectra for the reaction of the fully reduced wild-type fusion *Rs* aa3 with photoproduced O2 in the absence of CO. The intermediate spectra are referenced versus the original reduced enzyme. **Intermediate 1** is shown in blue, **Intermediate 2** in green, and **Intermediate 3** in red.
**Figure 14.** Comparison of the spectra of the first intermediates generated during O\textsubscript{2} reduction in either the absence or presence of CO in the wild-type fusion *Rs* *aa*\textsubscript{3}. The green curve represents the spectrum of **Intermediate 1** in the absence of CO (three-intermediate scheme); the spectrum of **Intermediate 1** was referenced versus **Intermediate 3**, the final intermediate (the oxidized enzyme) in the proposed wild-type fusion *Rs* *aa*\textsubscript{3} mechanism in the absence of CO. The blue curve represents the spectrum of the first intermediate, **R** (referenced versus the oxidized enzyme), generated during a conventional CO–O\textsubscript{2} flow-flash experiment (four-intermediate scheme) on the wild-type fusion *Rs* *aa*\textsubscript{3}. 
of CO (Figure 15, green) also agrees with the spectrum of Rs aa3 intermediate F in the presence of CO (Figure 15, blue). Figure 16 shows a proposed mechanism for the reaction between photoproduced O2 and the wild-type fusion Rs aa3 in the absence of CO.

The effective concentration of the photoproduced O2 is ~40 μM, and the apparent lifetime of 330 μs is associated with dioxygen binding. These values give rise to a second-order rate constant of ~8×10^7 M^{-1}s^{-1} for O2 binding in the absence of CO. The spectrum of compound A cannot be resolved due to the slow O2 binding, as mentioned above. When dioxygen binding slows down to 330 μs, the significantly faster conversion of A to F (53 μs in the conventional CO–O2 flow-flash experiment) prevents compound A from accumulating to detectable levels, and the next intermediate observed is F. The 2 ms lifetime observed during the reaction of the wild-type fusion Rs aa3 with photoproduced O2 in the absence of CO is attributed to the conversion of F to O, which is in agreement with the F to O transition (1.3 ms) during O2 reduction in the wild-type fusion Rs aa3 in the presence of CO (CO-O2 flow-flash experiment). These findings lend support to the results in Chapter 2, in which we observed the direct conversion of compound A to intermediate F (and not to P) in the O2 reduction mechanism of the wild-type fusion Rs aa3 in the presence of CO.
**Figure 15.** Comparison of the spectrum of the second intermediate generated during O$_2$ reduction in the absence of CO in the wild-type fusion *Rs aa$_3$* and the spectrum of F generated during O$_2$ reduction in the presence of CO. The green curve represents the spectrum of Intermediate 2 in the absence of CO, determined from a three-intermediate scheme, and is referenced versus Intermediate 3, the final intermediate (the oxidized enzyme) in the proposed wild-type fusion *Rs aa$_3$* mechanism in the absence of CO. The blue curve represents the spectrum of the third intermediate, F (referenced versus the oxidized enzyme), generated during a conventional CO–O$_2$ flow-flash experiment (four-intermediate scheme) on the wild-type fusion *Rs aa$_3$*. 
Figure 16. Proposed mechanism for the reaction between the fully reduced wild-type fusion Rs aa3 and photoproduced O2 in the absence of CO. Compound A, represented in brackets, is not observed.
Discussion

The second-order rate constants for O₂ and NO binding Rs aa₃ in the absence of CO are in agreement with the observed rates of O₂ and NO binding in bovine heart aa₃ (~1×10⁸ M⁻¹s⁻¹ for both) under the same conditions [17, 18]. However, O₂ and NO binding in the wild-type fusion Rs aa₃ is 10 times slower than in Tt ba₃, in the absence of CO [17, 18]. Crystallographic studies have suggested the presence of hydrophobic ligands channels connecting the binuclear center to the protein exterior in several heme-copper oxidases [23, 24]. Structural variations in the proposed ligand channels of Rs aa₃ and Tt ba₃ may contribute to this 10-fold kinetic difference [17, 18], and these are discussed in more detail below.

Structural differences in the proposed ligand channels of Rs aa₃ and Tt ba₃

The 10-fold difference in the ligand binding rates of Rs aa₃ and Tt ba₃ suggests inherent structural differences between the two enzymes. Based upon crystallographic studies involving pressurized xenon and krypton (which can gain access to hydrophobic pores within proteins), ligand channels have been proposed for Rs aa₃ and Tt ba₃ [23, 24]. The hydrophobicity of the channels allows the nonpolar O₂ and other ligands to diffuse through the protein to the catalytic site.

Figure 17 shows the proposed ligand channels in Rs aa₃ and Tt ba₃. The Tt ba₃ ligand channel is Y-shaped, with two possible entry/exit points at the protein/membrane interface, whereas the Rs aa₃ channel is one continuous pathway, with one entrance/exit [23, 24]. In Rs aa₃, two bulky amino acid residues—
Figure 17. A comparison of the proposed hydrophobic ligand channels in *Thermus thermophilus ba₃* (left) and *Rhodobacter sphaeroides aa₃* (right) based on crystallographic studies [23]. The putative "constriction point" residues are highlighted in the *Rs aa₃* enzyme (W172 and F282). The isopositional residues in *Tt ba₃* are also highlighted (Y133 and T231). Image from reference [23].
tryptophan 172 and phenylalanine 282 (Rs aa3 numbering)—protrude into the channel, forming a narrow section with a diameter of ~1.7 Å. In fact, there is a region of overlapping electron density between the W172 and F282 residues. This putative constriction point in Rs aa3 poses a structural barrier for ligands to overcome on their way to the binuclear center. It is probable that protein motions or conformational flexibility of the W172 and/or F282 residues, enable the passage of ligands to the active site. Classical molecular dynamics simulations in our laboratory have shown that residue F238 in the bovine enzyme (isopositional residue to F282 in Rs aa3) rotates out of the ligand channel, presumably not affecting ligand access to the active site. The narrow bottle-neck created by W172 and F282 may slow down ligand binding in Rs aa3. Chapter 4 discusses the Rs aa3 ligand channel in further detail.

The hydrophobic ligand pathway in Tt ba3 does not exhibit the narrowing of the channel seen in Rs aa3. The bulky Rs aa3 W172 and F282 residues (Rs aa3 numbering) are replaced with the less bulky tyrosine 133 and threonine 231 (Tt ba3 numbering), creating a wider channel in Tt ba3. The wider Tt ba3 ligand channel may serve to increase the rate of O₂ and NO diffusion to the active site. If the B-family of heme-copper oxidases evolved from the A-family [3, 11, 12], then the widening of the ligand channel may reflect an evolutionary adaptation to the native habitat of T. thermophilus. The T. thermophilus bacterium was originally isolated from a thermal vent in Japan [25] and can grow at temperatures as high as 82°C [25], where gas solubility is approximately half of that at room temperature [17, 23]. Moreover, the bacterium is found under anaerobic conditions, at oxic-anoxic interfaces, or in
hypersaline microbia mats [26]. Nature may have selected in favor of a more open ligand channel, which would maximize the rate of O₂ delivery to the binuclear center in Tt ba₃ under microaerobic environments.

The kinetics of O₂ and NO binding to the active site in the wild-type fusion Rs aa₃ were investigated in the absence and presence of CO. NO binds to the reduced heme a₃ with a lifetime of 100 μs, corresponding to a second-order rate constant of ~1×10⁸ M⁻¹s⁻¹ in the absence of CO. When CO is present, NO binds to heme a₃ with a second-order rate constant of ~9×10⁷ M⁻¹s⁻¹ in the presence of CO. The observed rates of O₂ and NO binding in the wild-type fusion Rs aa₃ are the same in presence and absence of CO, within experimental error. In contrast, the observed rates of ligand binding in Tt ba₃ are 10 times faster in the absence of CO (~1×10⁹ M⁻¹ s⁻¹) than in the presence of CO (~1×10⁸ M⁻¹ s⁻¹) [17, 18]. Thus, classical CO flow-flash experiments do not yield physiologically relevant information about the ligand binding dynamics in Tt ba₃. This raises the following question: why does the photodissociated CO alter the kinetics of O₂ and NO binding in Tt ba₃ but not in Rs aa₃? The cause of this phenomenon is unclear, but, as discussed below, it may be related to the transfer of CO to Cu₈ following photolysis of the heme a₃–CO bond.

**The lifetime of the Cu₈⁺–CO photoproduct in the aa₃ versus ba₃ oxidases**

Spectroscopic and mutational studies have indicated that Cu₈ may serve as an obligatory "way station" or gateway for ligands en route to and from the high-spin heme [27, 28]. Following laser-induced photolysis of the heme Fe²⁺–CO bond, CO
migrates to CuB, and the CuB⁺–CO photoproduct has been observed in bovine aa₃, Rs aa₃, and Tt ba₃ [29-31]. The lifetime of the CuB⁺–CO complex varies with the type of oxidase involved. In the bovine enzyme, the CuB⁺–CO photoproduct undergoes thermal dissociation at room temperature ($t_{1/2} \sim 1.5 \mu$s) [27]. Although low-temperature (253 K) FTIR experiments have indicated that CO binds to CuB for ~78 μs in Rs aa₃ [32], the lifetime of the Rs aa₃ CuB⁺–CO complex at room temperature is presumed to be consistent with that of the bovine enzyme. In contrast, the Tt ba₃ CuB⁺–CO photoproduct persists with a lifetime of ~30 ms—much longer than in the aa₃ enzymes. Tt ba₃ is the only known oxidase in which the binding of CO to CuB is exergonic [33, 34].

The present study reveals that O₂ and NO binding to the binuclear center in the wild-type fusion Rs aa₃ occurs on a timescale that does not overlap with the CuB⁺–CO photoproduct. Thus, CO does not restrict O₂ or NO from binding to the active site in the wild-type fusion Rs aa₃. This result is compatible with the theory that CuB may serve as a gate facilitating the diffusion of ligands to the active site.

However, is CuB a mandatory way-stop for ligands in Tt ba₃? The long lifetime of the Tt ba₃ CuB⁺–CO complex overlaps with the process of O₂ and NO binding, and appears to be in conflict with the CuB gateway proposal. If the Tt ba₃ active site can accommodate only one ligand at a time, then the longevity of the CuB⁺–CO complex would preclude O₂ and NO from binding to heme a₃. However, this scenario is not consistent with the fast rate of O₂ binding ($\sim 1 \times 10^8$ M⁻¹s⁻¹, 10 μs at 1 mM O₂) observed in TROA studies of Tt ba₃ with photoproduced O₂ in the
presence of CO [17]. Alternatively, if protein motions could enable two ligands to fit within the *Tt ba*$_3$ active site, then O$_2$ and NO could directly bind to heme $a_3$, bypassing the Cu$_B^+$–CO complex. However, this scenario does not involve a role for Cu$_B$ in directing ligands heme $a_3$.

The *Tt ba*$_3$ Cu$_B^+$–CO species also raises another conundrum: How is Cu$_B^+$ able to act as an electron donor during the fast dioxygen reduction if Cu$_B$ is complexed with CO on the millisecond timescale? The results were interpreted in terms of O$_2$ (or NO) binding to heme $a_3$ of *Tt ba*$_3$ triggering conformational or electrostatic changes in the Cu$_B$ environment, thus potentially altering the coordination geometry of Cu$_B$ [10]; these changes could potentially weaken the Cu$_B^+$–CO bond and promote CO to dissociate, thereby freeing Cu$_B$ to act as an electron donor during dioxygen reduction [10, 18]. It is possible that CO may remain on Cu$_B$ long enough to impede ligand binding in the *Tt ba*$_3$ enzyme (giving rise to the 10-fold slower rate in the presence of CO); however, the presence of O$_2$ in the ligand channel or at the heme $a_3$ site may trigger the early decay of the Cu$_B^+$–CO photoproduct, enabling Cu$_B$ to donate electrons during catalysis.

**Crystallographic studies of the bovine *aa*$_3$ and *Tt ba*$_3$ Cu$_B^+$–CO photoproducts**

A comparison of the pre- and post-photolysis crystal structures of the bovine *aa*$_3$ and *Tt ba*$_3$ enzymes provides insight into the CO binding dynamics at the active site following photodissociation of CO from heme $a_3$. Although crystal structures of the CO-bound *Rs aa*$_3$ before and after photolysis are not currently available, the
bovine aa3 structures offer a starting point from which to compare the aa3 and ba3 enzymes.

In the "dark" pre-photolysis bovine crystal structure, CO is oriented almost perpendicular to the plane of heme a3, with a Fe–C–O angle of ~164°, and a heme a3 Fe–C bond of ~1.8 Å (Figure 18, top) [35]. In the "light" post-photolysis crystal structure of the bovine CuB⁺–CO photoproduct (Figure 18, bottom), CO adopts a "side-on" orientation with respect to CuB, with CuB ~2.4 Å away from C, and ~2.7 Å away from O [35]. These values indicate that the association of CuB and CO is very weak in the bovine heart aa3 photoproduct. The C atom of the CO is displaced 3.0 Å away from the heme a3 iron [35]. In the "dark" structure, CO is positioned directly above the heme a3 iron; however, in the "light" structure, CO is shifted away from the heme a3 iron, closer to the entrance of the postulated ligand channel [10].

In the "dark" Tt ba3 crystal structure preceding photolysis, CO is oriented directly above the heme a3 iron, with a Fe–C–O angle of ~126° (Figure 19, top) [36]. The heme a3 Fe–C bond is ~1.95 Å, and the distance between O and CuB is ~2.42 Å. In the CuB⁺–CO photoproduct (the "light" structure) (Figure 19, bottom), the CuB⁺–CO bond length is ~1.9 Å, and the distance between the heme a3 iron and O is ~2.3 Å [36]. This suggests that CO is bound more tightly to CuB in Tt ba3 than in bovine aa3.

The post-photolysis bovine structure indicates that CO has shifted from its pre-photolysis position above the heme a3 iron, and CO may be on a trajectory out of the binuclear center towards the entrance to the proposed ligand channel. However,
Figure 18. Comparison of the pre- and post-photolysis crystal structures of the CO-bound bovine heart \textit{aa}_3. Top: The pre-photolysis CO-bound bovine \textit{aa}_3 (PDB 3AG1). Bottom: The post-photolysis CO-bound bovine \textit{aa}_3 (PDB 3AG2). Image generated using Pymol.
Figure 19. Comparison of the pre- and post-photolysis crystal structures of the CO-bound *T. thermophilus* ba$_3$. Top: The pre-photolysis CO-bound *Tt* ba$_3$ (PDB 3QJQ). Bottom: The post-photolysis CO-bound *Tt* ba$_3$ (PDB 3QJR). Image generated using Pymol.
following CO photodissociation in *Tt* ba3, CO does not appear to follow a trajectory directly out of the active site; rather, CO appears to rotate in place within the binuclear center, staying directly above the heme *a*3 iron. This behavior may explain why CO impedes ligand binding in *Tt* ba3 but not in the *aa*3-type oxidases.

**Conclusions**

In the absence of CO, the second-order rate constants of O2 and NO binding to heme *a*3 in the wild-type fusion *Rs* aa3 are 10 times smaller than in *Tt* ba3. This dramatic disparity in kinetic behavior may originate from structural differences in the postulated ligand channels of the *Rs* aa3 and *Tt* ba3 enzymes. Indeed, a crystallographic comparison of the two enzymes reveals that *Tt* ba3 has a relatively open ligand pathway, whereas a narrowing of the proposed ligand channel in *Rs* aa3 may restrict ligand access to the binuclear center. The steric barrier created by residues W172 and F2821 in the *Rs* aa3 enzyme may contribute to the slower rate of O2 and NO binding in the absence of CO in this enzyme.

The present work also reveals that the presence of CO does not impede O2 and NO binding to the active site in the wild-type fusion *Rs* aa3. The second-order rate constants of O2 and NO binding to heme *a*3 are essentially the same in the presence and absence of CO (≈1×10⁸ M⁻¹s⁻¹ in the presence and absence of CO). This result contrasts with the kinetic behavior of the *Tt* ba3 enzyme, which experiences a 10-fold

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1 Residue F282 in the *Rs* aa3 enzyme may exhibit a degree of conformational flexibility. As suggested by molecular dynamics simulations in our laboratory, in the bovine heart enzyme, the isopositional residue, F238, has conformational flexibility and is able to rotate out of the proposed ligand channel.
slow-down in the rate of O₂ and NO binding in the presence of CO (≈1×10⁹ M⁻¹s⁻¹ in the absence of CO vs. ≈1×10⁸ M⁻¹s⁻¹ in the presence of CO). In bovine aa₃, the photolyzed CO may adopt a trajectory leading out of the active site and towards the entrance of the ligand channel. The post-photolysis bovine crystal structure shows that the photolyzed CO is already "escaping" the binuclear center; this behavior may explain why the presence of CO does not have an observable impact on O₂ and NO binding in the highly homologous aa₃-type oxidase from *Rhodobacter sphaeroides*. In contrast, in the *Tt ba₃* post-photolysis product the presence of CO directly above the heme a₃ iron may sterically restrict the binding of O₂ and NO in *Tt ba₃*; this may cause the ligand binding to slow down by a factor of 10 in the presence of CO.

The lifetimes of the *Rs aa₃* and *Tt ba₃* CuB⁺–CO photoproducts were also considered within the context of the "gateway" theory for CuB. The short lifetime of the *Rs aa₃* CuB⁺–CO photoproduct does not overlap with the timescale of ligand binding to heme a₃, and CuB is available to transiently bind ligands on their path to heme a₃ and serve as an electron donor during catalysis. However, the unusually long lifetime of the *Tt ba₃* CuB⁺–CO photoproduct poses a challenge to the idea that CuB functions as a way-stop for ligands en route to the active site. An alternative scenario—involving the transient binding of two ligands at the binuclear center—has been proposed for *Tt ba₃* [10]. Ultimately, the proposal that ligands like O₂ and NO transiently bind CuB prior to reaching the active site may not be relevant for all heme-copper oxidases.
References


Chapter 4

Spectral Investigation of the W172Y/F282T and G283L Ligand Channel Mutants of the \textit{aa}_3 \textit{Rhodobacter sphaeroides} Cytochrome \textit{c} Oxidase
Introduction

Molecular oxygen is a necessary substrate in numerous biological reactions involving oxidases, oxygenases, and flavoenzymes [1-9]. For catalysis to occur, enzymes must bring O$_2$ through the protein matrix to a metal prosthetic group(s) or active site, often buried deep inside the protein. However, little is known about the mechanisms by which oxygen reaches the catalytic site.

Historically, the prevailing theory proposed that dioxygen could directly enter and move freely through the protein matrix via "liquid-like diffusion" rather than specialized intraprotein channels [10]. Experimental evidence that challenges this hypothesis has recently emerged. Crystallographic studies have identified hydrophobic channels in several enzymes, including heme-copper oxidases from *Rhodobacter sphaeroides (Rs aa$_3$)* [8], bovine heart *aa$_3$* [7], *Paracoccus denitrificans (Pd aa$_3$)* [5], *Thermus thermophilus (Tt ba$_3$)* [11], and *Paracoccus stutzeri cbb$_3$* [12]. The proposed channels may serve as routes for the diffusion of dioxygen (as well as other ligands), and may modulate catalysis by moderating O$_2$ access to the active site.

Time-resolved optical absorption (TROA) spectroscopy measurements in our laboratory have shown that O$_2$ and NO bind to reduced *Thermus thermophilus ba$_3$ (Tt ba$_3$)* cytochrome oxidase with a superfast second-order rate constant of $1 \times 10^9$ M$^{-1}$s$^{-1}$, which is 10 times faster than observed for the bovine enzyme and *Rs aa$_3$* [13, 14]. Crystallographic studies have shown inherent structural differences in the ligand channels of the *aa$_3$* and *ba$_3$* oxidases that may account for this 10-fold difference [15]. In *Rs aa$_3$*, bovine *aa$_3$*, and *Pd aa$_3$*, two bulky amino acid residues, tryptophan and
phenylalanine (W172 and F282 in Rs aa3), in the catalytic subunit I, constrict the ligand channel ~9 Å from the binuclear center [16-18], while in Tt ba3, smaller residues, tyrosine (Y133) and threonine (T231), occupy the corresponding sites [11, 19] (Figure 1). The bulky W172 and F282 residues in the aa3 oxidases have been proposed to slow down access to the active site, thereby giving rise to a reduced rate of ligand binding [19]. Recent time-resolved optical absorption studies in our laboratory of the Tt ba3 Y133W and Y133W/T231F mutants resulted in 5 times slower rate of O2 and NO binding than in the wild-type enzyme; however, the ligand binding rate was unaffected in the T231F mutant [20]. As shown by classical molecular dynamics calculations of Xe and O2 diffusion to the active site of the Y133W/T231F mutant, this is because of conformational freedom of the F231 side chain, which rotates out of the ligand channel, with no effect on ligand access. This is also true for the side chain of the homologous F238 in the bovine enzyme [20]. The effect of replacing simultaneously the bulkier W172 and F282 “constriction” residues in Rs aa3 by the homologous smaller residues present in Tt ba3 has not been investigated.

Also located within the proposed ligand channel in Rs aa3 is a highly conserved glycine residue (G283) immediately adjacent to the putative constriction point defined by W172 and F282, described above [15]. G283 is ~8 Å from the heme a3 iron and ~6 Å away from the constriction point [8]. Due to the absence of a side chain on the glycine residue, G283 may serve as an entry point for ligands to gain access to the binuclear center.
Figure 1. A comparison of the proposed hydrophobic ligand channels in *Thermus thermophilus* ba₃ (left) and *Rhodobacter sphaeroides* aa₃ (right). Image from reference [15].
Flash-photolysis of CO, a competitive inhibitor of O₂, from the reduced CO-bound wild-type and mutant heme-copper oxidases has frequently been used to investigate the kinetics of ligand binding in these enzymes (for review, see ref. [21]). Previous Fourier transform infrared (FTIR) and time-resolved infrared (TRIR) experiments have provided strong support for CO binding to CuB⁺ following CO photolysis from the high-spin heme a₃ in the heme-copper oxidases [21-29]. Transient optical absorption studies of CO recombination as a function of CO have also provided support for the transient binding of CO to CuB⁺ from solution en route to the high-spin heme [25, 30]. Transient optical absorption measurements have shown that CO recombines with heme a₃ in the bovine and Rs aa₃ oxidases on 10-20 ms time scale [25, 31].

In this study, we investigated the roles of W172 and F282 in modulating ligand access to the active site in Rs aa₃. We examined the CO recombination kinetics following photolysis of the fully reduced CO-bound W172Y/F282T double mutant, in which the W172 and F282 residues in the ligand channel of Rs aa₃ were replaced with the smaller residues, tyrosine and threonine, respectively, mimicking the ligand channel in Tt ba₃. The presence of multiple enzyme conformers was observed. The results suggest that the double mutation closes the exit channel, resulting in ~500-fold increase in the rate of CO recombination compared to the wild-type enzyme.

We also investigated the role of G283 in moderating the access of ligands to the active site in Rs aa₃ by mutating glycine to the larger leucine residue (Figure 2). This mutation introduces additional steric bulk in the proposed ligand channel of
**Figure 2.** The amino acid structures of glycine (left) and leucine (right). Image prepared using ChemSketch, version 14.01.
The enzyme activity, oxidation rate, and ground state optical absorption spectrum of the G283L mutant were determined, and the CO photodissociation and recombination kinetics were measured by TROA spectroscopy. The results suggest that enzymatic turnover is compromised in the G283L Rs aa₃ mutant, and multiple CO conformers are present in enzyme preparations of the G283L mutant. In one particular enzyme conformer, the CO recombination rate in the G283L mutant is ~500 times faster than in the wild-type Rs aa₃.

Materials and Methods

The W172Y/F282T R. sphaeroides strain, the pJS3-SH and pRK415 plasmids, E. coli S-17-1 strain, and the R. sphaeroides JS100 cell strain were generously provided by Professor Robert Gennis, University of Illinois at Urbana-Champaign. All chemicals used were of the highest purity reagent grade. The detergent, n-dodecyl-β-D-maltopyranoside (DM), was obtained from Anatrace. Ni-NTA resin was obtained from Qiagen. Standard grade (99.9%) carbon monoxide was purchased from Praxair. All other chemicals were from Fisher Biosciences and Sigma-Aldrich unless otherwise noted.

Construction of the subunit I G283L mutant of Rs aa₃

The QuikChange site-directed mutagenesis kit (Agilent Technologies) was used to introduce the G283L mutation into a template plasmid, pJS3-SH [32-34]. Plasmid pJS3-SH contains a gene for ampicillin resistance, as well as the His-tagged
ctaD gene, which encodes Rs aa3 subunit I (Figure 3) [35]. Figure 4 shows the oligonucleotide primers used in the mutagenesis reaction; primers were synthesized at Elim Biopharmaceuticals (Hayward, CA). The 25 µL mutagenesis reaction mixture contained the following: 10 ng of pJS3-SH (in 1 µL); 0.4 µL of the 20 µM forward primer; 0.4 µL of the 20 µM reverse primer; 2.5 µL of 10X reaction buffer; 1 µL of dNTP mix; 1.5 µL of QuikSolution; 16.7 µL of nuclease-free water (Zymo Research); 1 µL of MgCl2; and 0.5 µL of PfuUltra high fidelity DNA polymerase. A PTC-100 Programmable Thermal Controller (MJ Research) was used for the mutagenesis. An initial melting step at 98°C for 3 min was performed, followed by a total of 18 cycles, each consisting of a 30 s denaturation phase at 98°C, a 50 s annealing phase at 57°C, and a 6 min extension phase at 68°C. A final 10 minute extension at 68°C was also included. Next, 0.5 µL of DpnI (supplied with the kit), a restriction enzyme which only cleaves at methylated sites, was added directly to the reaction mixture and thoroughly mixed; the mixture was subsequently incubated overnight at 37°C to selectively digest the parental (non-mutated) DNA and retain the mutagenized DNA. The mutated DNA was purified using a DNA Clean & Concentrator™ Kit (Zymo Research) and eluted in 15 µL of nuclease-free water. The purified DNA was used to transform XL-10 Gold Ultracompetent cells (provided with the kit), according to the manufacturer's instructions. The transformed cells were spread on LB agar plates containing 100 µg/mL ampicillin (final concentration) and incubated overnight at 37°C. Ampicillin-resistant colonies were picked and used to inoculate 5 mL of LB media (100 µg/mL ampicillin, final concentration); the bacteria were cultured in an
Figure 3. Simplified plasmid map of plasmid pJS3-SH.
Forward primer (5' to 3'): CTG TGG TTC TTC CTG CAC CCG GAG GTC
Reverse primer (5’to 3’): GAC CTC CGG GTG CAG GAA GAA CCA CAG

**Figure 4.** The sequences of the forward and reverse primers used for site-directed mutagenesis during construction of the G283L mutant of *Rs aa3*. The targeted mutation is shown in red.
The mutagenized plasmid was extracted using the QIAprep Spin Miniprep Kit (Qiagen) and eluted in 20 μL nuclease-free water. The purified plasmid was sequenced by the University of Illinois at Urbana-Champaign (UIUC) Biotechnology Center (Urbana, IL) for verification of the desired mutation. An abbreviated workflow is shown in Figure 5.

The mutagenized pJS3-SH plasmid and the broad-host range expression vector, pRK-415 (Figure 6) [36], were digested with EcoRI and HindIII (both from New England BioLabs) for 2.5 hours at 37°C, and the digestion fragments were separated by 0.8% agarose gel electrophoresis (Tris-acetate-EDTA buffer, 60 volts for 1 hour and 15 minutes). The ~1.9 kb fragment from pJS3-SH (the insert), which contains the His-tagged CcO subunit I, and the ~10.5 kb fragment from pRK-415 (the vector), which contains a tetracycline resistance gene, were purified via gel extraction using the Zymolean™ Gel DNA Recovery Kit (Zymo Research). The ~1.9 kb fragment from pJS3-SH was ligated into the ~10.5 kb fragment from pRK415. The 10 μL ligation reaction mixture contained the following: 1 μL of T4 DNA ligase (New England BioLabs), 1 μL of 10X T4 DNA ligase reaction buffer (containing ATP); 8 μL of nuclease-free water; ~50 ng of vector (the pRK-415 fragment); and ~150 ng of insert (the pJS3-SH fragment). The ligation reaction mixture was incubated overnight at 16°C. The entire ligation reaction was used to transform Top10 Chemically Competent cells (Invitrogen). The transformed cells were spread on LB agar plates containing 1 μg/mL tetracycline (final concentration) and incubated overnight at 37°C. Tetracycline-resistant colonies were picked and used to inoculate 5 mL LB media
Figure 5. Workflow summarizing the steps for site-directed mutagenesis and cell transformation.
Figure 6. Simplified plasmid map of the broad-host range expression vector, pRK-415.
(1 μg/mL tetracycline, final concentration); bacteria were cultured in an incubator-shaker overnight at 37°C. The pRK415 expression plasmid harboring the G283L mutation was extracted using the QIAprep Spin Miniprep Kit (Qiagen) and eluted in 20 μL of nuclease-free water. The purified plasmid was sequenced by Elim Biopharmaceuticals (Hayward, CA) for verification of the desired mutation. The restriction digest, ligation, and transformation steps are summarized in Figure 7.

Bacterial conjugation between *E. coli* S-17-1 and *R. sphaeroides* JS100 was performed in order to generate a strain of *R. sphaeroides* which expressed the G283L mutation in subunit I of CcO. The *E. coli* S-17-1 bacterial strain served as a donor to transfer the mutated pRK415 expression plasmid into *R. sphaeroides*. The *R. sphaeroides* JS100 strain has been engineered with a deletion of CcO subunit I in its genome, which is complemented by expression of subunit I encoded by the mutated pRK415 expression plasmid; the JS100 strain also contains resistance genes for streptomycin and spectinomycin [35]. The Z-competent *E. coli* Transformation Kit (Zymo Research) was used to make *E. coli* S-17-1 cells chemically competent, and the pRK415 plasmid containing the *ctaD* gene was used to transform the *E. coli* S-17-1 cells. *R. sphaeroides* JS100 cells were inoculated in Sistrom’s minimal media [37, 38] containing 50 μg/mL spectinomycin and 50 μg/mL streptomycin (final concentrations) and grown to early log phase. The transformed *E. coli* S-17-1 (100 μL) and *R. sphaeroides* JS100 (1 mL) cells were spun down separately in sterile microcentrifuge tubes (3000 RPM, 5 minutes). The cell pellets were washed 3 times in 1 mL of sterile LB broth (to wash out any remaining antibiotics) and combined
Figure 7. Workflow summarizing the steps for the restriction digest, ligation, and subsequent cell transformation.
in 100 µL of sterile LB broth. The combined cell suspension was pipetted on top of a sterile 0.45 μm Millipore filter placed upon a LB agar plate without antibiotics. For ~30 minutes, excess liquid was allowed to diffuse through the filter and into the agar, leaving the *E. coli* S-17-1 and *R. sphaeroides* JS100 cells atop the filter. The plated cells were incubated overnight at 30°C for bacterial conjugation. Sterile tweezers were used to remove the Millipore filter and place it in a microcentrifuge tube containing 1 mL of sterile Sistrom’s minimal media. The tube was vortexed briefly to rinse the cell paste off the filter, and 50 µL of the cell suspension was diluted with 100 mL of Sistrom's minimal media. 100 µL of the diluted cell suspension was spread on an agar plate containing Sistrom's minimal media, 50 µg/mL streptomycin, 50 µg/mL spectinomycin, and 1 µg/mL tetracycline (final concentrations). The plated cells were incubated at 30°C for ~4 days. Colonies were picked and restreaked on fresh Sistrom's plates containing 50 µg/mL streptomycin, 50 µg/mL spectinomycin, and 1 µg/mL tetracycline (final concentrations). Resulting colonies that formed were inoculated in 5 mL of Sistrom's minimal media containing the three antibiotics, the plasmid was extracted, and sequencing was performed by Elim Biopharmaceuticals to verify the presence of the desired mutation (Figure 8). Once verified, the *R. sphaeroides* transconjugants were used to express the G283L mutant of *R s aa3*.

**Growth of bacteria and enzyme purification**

All *R. sphaeroides* strains were grown aerobically in the dark, in Sistrom's minimal media (50 µg/mL tetracycline, 50 µg/mL spectinomycin, and 1 µg/mL
**Figure 8.** Partial DNA sequence alignment verifying the presence of the G283L mutation in *Rs aa3*. The codon for glycine is GGC (shown in red), and the codon for leucine is CTG (shown in blue).
streptomycin (final concentrations) as previously described in the Materials and Methods section of Chapter 2. The His-tagged variants of CcO were isolated and purified according to the protocol outlined in Chapter 2.

*Enzyme activity assays of the wild-type and G283L mutant of Rs aa₃ CcO*

Enzyme activity measurements of the wild-type and G283L mutant of *Rs aa₃* were performed by monitoring the oxidation of reduced horse heart cytochrome *c* by cytochrome *c* oxidase at 550 nm. A 1 µM solution of the appropriate CcO variant was made in 50 mM sodium phosphate buffer (pH 7.5). A solution of horse heart cytochrome *c* in 50 mM sodium phosphate buffer (pH 7.5) was reduced by adding three small scoops of solid sodium dithionite. To remove excess dithionite, the reduced cytochrome *c* solution was applied to the top of a PD-10 Sephadex G-25M column, which had been equilibrated with 50 mM sodium phosphate buffer (pH 7.5). The reduced cytochrome *c* solution was allowed to flow through the column via gravity, enabling the separation of the cytochrome *c* and dithionite. The reduced cytochrome *c* was eluted from the column, and its absorbance spectrum was recorded on a Hewlett-Packard 8453 UV/Vis spectrophotometer. The concentration of reduced cytochrome *c* was determined using an extinction coefficient of $\varepsilon = 30.4 \text{ mM}^{-1}\text{cm}^{-1}$ at 550 nm [39]. Subsequently, 380 µL of 15 µM reduced cytochrome *c* were added to 610 µL of sodium phosphate buffer (pH 7.5) in a cuvette, and 10 µL of a 1 µM CcO solution (in sodium phosphate buffer, pH 7.5) were added. The cuvette was inverted to mix the reagents and placed in a spectrophotometer. The oxidation of cytochrome *c*
at 550 nm was monitored for 6 – 50 seconds in the kinetic mode. In order to
determine the end point of the reaction, a minimal amount of solid ferricyanide was
added to the cuvette to fully oxidize any remaining reduced cytochrome c. The rate
constant was calculated according to the method of Smith [40]. The enzyme activity
was determined by dividing the rate constant by the effective CcO concentration in
the cuvette.

**CO flash-photolysis measurements**

Each Rs aa₃ enzyme variant was diluted in a 50 mM sodium phosphate
supplemented with 0.1% DM (pH 7.4). The enzyme sample was deoxygenated by
several cycles of evacuation on a vacuum line, followed by flushing with inert
nitrogen gas.

The sample was reduced under N₂ gas with a few grains of dithionite or a mixture
of sodium ascorbate (1 mM, final concentration) and the redox-mediator, ruthenium
hexamine chloride (0.5 μM, final concentration). The reduced sample was exposed to
CO gas for 15 to 20 minutes at room temperature with occasional agitation to
generate the reduced CO-bound enzyme.

The CO recombination following photolysis of the reduced CO-bound Rs aa₃
enzyme (wild-type, G283L, or W172Y/F282T variant) was investigated using time-
resolved optical absorption (TROA) spectroscopy as previously described [31, 41].
Briefly, the CO ligand was photolyzed by a 532 nm laser flash (DCR-11 Nd:YAG, 30
mJ per pulse, 7-ns duration). TROA spectra were recorded in the 350 - 700 nm region
over a time period from 200 ns to 500 ms following CO photolysis. The probe beam, a high-power Xenon flash lamp, was passed through the sample and filters and then focused onto a spectrograph. The TROA signals were recorded by a CCD camera before and after the laser flash and difference spectra (post- minus pre-photolysis) calculated. The TROA difference spectra were analyzed by singular value decomposition (SVD) and global exponential fitting [42-45], and the spectral changes and lifetimes of the CO recombination in the wild-type, G283L, and W172Y/F282T enzymes were compared.

**Results**

*The spectrum of the as-isolated G283L mutant of Rs aa₃*

Figure 9 (green curve) shows the ground state optical absorption spectrum of the "as-isolated" G283L enzyme (immediately after elution from the nickel column); the spectrum exhibits two absorption maxima in the Soret region (425 and 441 nm), as well as an α-band at 604 nm. In contrast, the spectrum of the "as-isolated" wild-type enzyme (Figure 9, blue curve) displays only one Soret band at 424 nm, as well as a 599 nm peak in the visible region. According to previously published spectra, the absorption maxima are 423 nm and 599 nm for the oxidized wild-type Rs aa₃, and 443 nm and 604 nm for the reduced wild-type enzyme [46]. The spectrum of the ascorbate-reduced G283L enzyme contains a 442 nm peak in the Soret region, and a 604 nm peak in the visible region (Figure 10).
Figure 9. The optical absorption spectra of the as-isolated G283L Rs aa3 mutant and the wild-type Rs aa3. The G283L Rs aa3 is shown in green and the wild-type Rs aa3 is shown in blue.
Figure 10. The optical absorption spectra of oxidized and reduced G283L Rs aa$_3$.

The spectrum of the oxidized G283L Rs aa$_3$ is shown in blue and the spectrum of the reduced G283L Rs aa$_3$ is shown in green.
Spectrophotometric assays of the G283L Rs aa₃

The oxidation of cytochrome c by the wild-type or G283L Rs aa₃ was measured according to the method of Smith [40] (data not shown). The measured enzyme activity of the G283L mutant was ~50% lower than observed in the wild-type Rs aa₃ (~2.5×10⁻⁶ s⁻¹M⁻¹ for the wild-type versus ~1.2×10⁻⁶ s⁻¹M⁻¹ for the G283L).

The oxidation of the reduced G283L enzyme by dioxygen was measured according to reference [47], except the absorbance change at 425 nm was monitored with respect to time. Figure 11 shows the kinetic trace of the oxidation of the reduced G283L mutant. Upon exposure to dioxygen, the oxidation of the reduced G283L mutant is significantly slower (on the order of minutes) than in the wild-type Rs aa₃ (~1 ms) [48].

CO photodissociation and recombination in the wild-type and G283L Rs aa₃

The CO photodissociation and recombination kinetics of the wild-type and G283L Rs aa₃ enzymes were investigated by multi-wavelength TROA spectroscopy. CO photolysis of the reduced CO-bound enzymes was accomplished by a 532 nm laser pulse, and the difference spectra (post- minus pre-photolysis) were recorded between 200 ns – 500 ms after the laser flash. Figure 12 shows the TROA difference spectra (post- minus pre-photolysis) acquired during the CO flash-photolysis experiment of the reduced CO-bound wild-type Rs aa₃. Global exponential fitting of the SVD-filtered TROA data [41, 44, 45] resolved four apparent lifetimes for the wild-type Rs aa₃: 0.9 μs, 54 μs, 500 μs, and 23 ms. The wild-type b-spectra are shown
Figure 11. The oxidation of reduced G283L Rs aa$_3$ upon exposure to dioxygen
Figure 12. TROA difference spectra (post- minus pre-photolysis) recorded in the Soret and visible regions following photolysis of the fully reduced CO-bound wild-type Rs aa3.
in Figure 13A. The recombination of CO to heme $a_3$ is attributed to the highest amplitude $b$-spectrum ($b_4$, 23 ms). The residuals are shown in Figure 13B.

Figure 14 shows the TROA difference spectra (post- minus pre-photolysis) acquired during the CO flash-photolysis experiment of the reduced CO-bound G283L mutant. Three exponentials were sufficient to fit the data, and the apparent lifetimes were 49 $\mu$s, 487 $\mu$s, and 9.4 ms. The G283L $b$-spectra and residuals are shown in Figure 15A and 15B, respectively. The amplitudes of the individual $b$-spectra are similar in size, suggesting the presence of multiple enzymes populations which are capable of rebinding CO after photolysis.

**CO photodissociation and recombination in the W172Y/F282T Rs aa$_3$ mutant**

In *Rs aa$_3$*, two bulky residues in subunit I, W172 and F282, create a narrow constriction point in the ligand channel that connects the protein exterior to the buried active site (Figure 1). To mimic the wider *Tt ba$_3$* ligand channel, a double mutant of *Rs aa$_3$* was created in which the W172 and F282 residues were replaced by tyrosine and threonine, respectively, creating the W172Y/F282T double mutant. To investigate the CO recombination kinetics in the W172Y/F282T double mutant and wild-type *Rs aa$_3$*, the reduced CO-bound enzyme complexes were photolyzed by a 532 nm laser pulse and the difference spectra (post- minus pre-photolysis) recorded between 200 ns – 500 ms after CO photolysis (Figure 16). The TROA spectra have been normalized to the same concentration based on the absolute spectra of the oxidized enzymes.
Figure 13. The $b$-spectra (Panel A) and residuals (Panel B) resulting from a four-exponential fit of the SVD-filtered TROA data following photolysis of the fully reduced CO-bound wild-type Rs $aa_3$. 
**Figure 14.** TROA difference spectra (post- minus pre-photolysis) recorded in the Soret and visible regions following photolysis of the fully reduced CO-bound G283L mutant of *Rs aa3.*
Figure 15. The $b$-spectra (Panel A) and residuals (Panel B) resulting from a three-exponential fit of the SVD-filtered TROA data following photolysis of the fully reduced CO-bound G283L mutant of $Rs\; aa_{3}$.
Figure 16. TROA difference spectra (post- minus pre-photolysis) recorded in the Soret and visible regions following photolysis of the fully reduced CO-bound W172Y/F282T mutant of Rs aa3.
The TROA difference spectra were analyzed by SVD and global exponential fitting [41, 44, 45]. Figure 17 shows the $b$-spectra and residuals for the W172Y/F282T mutant. A three-exponential fit of the data for the W172Y/F282T mutant yielded apparent lifetimes of 0.6 µs, 44 µs, and 300 µs. The processes corresponding to the highest amplitude $b$-spectra, 23 ms in the wild-type enzyme (Figure 13A) and 44 µs for the mutant (Figure 17A), are attributed to the CO recombination to heme $a_3$.

**Discussion**

**Air-oxidation, enzyme activity, and spectral properties of the G283L Rs aa$_3$**

The air-oxidation of the reduced G283L mutant was slowed by several orders of magnitude compared to the wild-type enzyme, suggesting that the larger leucine residue effectively restricts $O_2$ access to the binuclear center. Replacement of G283 with leucine reduced enzyme activity by ~50%. Furthermore, the spectrum of the as-isolated G283L mutant suggests that hemes $a$ and $a_3$ are not fully oxidized (Figure 9, green). After enzyme purification, the wild-type Rs aa$_3$ is typically in the oxidized form (Figure 9, blue). The catalytic reduction of dioxygen to water results in the formation of oxidized CcO, and the enzyme remains trapped in the oxidized state if no reducing equivalents are available. Because heme $a$ is the primary absorber in the visible region [49], the 605 nm $\alpha$-band in the spectrum of the as-isolated G283L enzyme suggests the presence of reduced heme $a$. Heme $a_3$ on the other hand, is the primary absorber in the Soret region [49]; the peaks at 425 nm and 441 nm in the
Figure 17. The \( b \)-spectra (Panel A) and residuals (Panel B) resulting from a three-exponential fit of the SVD-filtered W172Y/F282T TROA data following photolysis of the fully reduced CO-bound W172Y/F282T mutant of \( Rs \) aa3.
G283L spectrum are similar in amplitude, suggesting a mixture of reduced and oxidized heme $a_3$. The partially-reduced nature of the as-isolated G283L mutant may indicate that a significant enzyme population cannot complete a full catalytic cycle; this may be due to structural changes resulting from the G283L mutation. For example, the G283L mutation may disrupt the D-channel H-bond network near the binuclear center.

The $aa_3$-type heme-copper oxidases contain proton-conducting D- and K-paths [33, 50-53]. To achieve unidirectional proton pumping from the negative side to the positive side of the inner mitochondrial or bacterial plasma membrane, CcO presumably contains a protonable functional group which can bind a proton from the N-side, and release the proton to the P-side [54, 55]. This proton-loading site (PLS) forms the basis of a gating mechanism for proton pumping in CcO. The gating region is purported to be in the vicinity of E286 ($R. sphaeroides$ numbering) [56, 57], a highly conserved D-pathway residue near the binuclear center. In order to prevent the nonspecific back flow of protons (back leaks), the protein structure in the vicinity of the gating machinery must be rigid. The conserved G283 residue is located in the proposed ligand channel of $R. sphaeroides$ [8] and is ~4 Å away from E286, in an area where the proposed ligand channel and gating region overlap.

The crystal structure of the wild-type Rs $aa_3$ shows a hydrogen bond between the backbone carbonyl oxygen of G283 and a water molecule ~3.2 Å away (Figure 18) [58]; this water molecule (designated as water #301) is in turn H-bonded to an OH$^-$ ligand of Cu$_B$. It has been postulated that water 301 may serve as a connecting bridge
Figure 18. The location of a water molecule (W301, red sphere) and a Cu$_B$ OH$^-$ ligand (cyan sphere) in the X-ray crystal structure of Rs $aa_3$ (PDB 2GSM). Iron and Cu$_B$ are shown in gray and gold, respectively.
from E286 to the PLS and the binuclear center [59]; by forming a H-bond with the carbonyl oxygen of E286 when this residue adopts a possible "up" conformation [57, 60, 61]. On the other hand, the replacement of glycine 283 with the bulkier leucine residue may sterically disrupt the H-bond which holds a water molecule in place between E286 and the PLS or BNC. This could potentially hinder the transfer of D-channel protons and may restrict enzyme turnover in the G283L mutant.

**CO photodissociation and recombination in the G283L Rs aa₃ mutant**

To probe the role of the highly conserved G283 residue in the proposed ligand channel of Rs aa₃, the CO recombination kinetics of the wild-type enzyme and G283L mutant were investigated. The spectroscopic signatures of the unliganded and CO-bound CcO are distinct, and CO recombination can be monitored using time-resolved optical absorbance spectroscopy. The SVD-filtered TROA spectra acquired during the G283L CO flash-photolysis experiment were best fit with three lifetimes, whereas four lifetimes were required to fit the wild-type Rs aa₃ TROA data. The first lifetime for the G283L mutant (49 μs) is similar to the second lifetime of the wild-type enzyme (54 μs), and the corresponding b-spectra for both enzymes are in good agreement. Figure 19 shows a one-to-one comparison of the G283L b₁-spectrum with the wild-type b₂-spectrum. As shown in Figure 20, the G283L b₂-spectrum (487 μs) is consistent with the wild-type b₃-spectrum (500 μs). The spectral comparison in Figure 21 shows that the b₃-spectrum of the G283L mutant (9.4 ms) is similar to the wild-type b₄-spectrum (23 ms).
**Figure 19.** A comparison of the G283L *Rs aa*$_{3}$ $b_1$-spectrum with the wild-type *Rs aa*$_{3}$ $b_2$-spectrum. The G283L $b_1$-spectrum is shown in blue, and the wild-type $b_2$-spectrum is shown in green. The wild-type $b_2$-spectrum has been multiplied by a factor of 2.4 to normalize it to the G283L $b_1$-spectrum.
**Figure 20.** A comparison of the G283L Rs aa3 b2-spectrum with the wild-type Rs aa3 b3-spectrum. The G283L b2-spectrum is shown in blue, and the wild-type b3-spectrum is shown in green. The wild-type b3-spectrum has been multiplied by a factor of 3.3 to normalize it to the G283L b2-spectrum.
Figure 21. A comparison of the G283L Rs aa₃ b₃-spectrum with the wild-type Rs aa₃ b₄-spectrum. The G283L b₃-spectrum is shown in blue, and the wild-type b₄-spectrum is shown in green. The wild-type b₄-spectrum has been multiplied by a factor of 0.145 to normalize it to the G283L b₃-spectrum.
The spectral data indicate the presence of different conformers of the wild-type and G283L Rs aa3 enzymes. The presence of CcO conformers is consistent with previous resonance Raman and FTIR studies of CcO from *R. sphaeroides* [62, 63]. The large amplitude of the wild-type *b*4-spectrum (Figure 13A) suggests that this conformer is the most abundant, with a CO recombination lifetime of 23 ms. However, the G283L *b*-spectra are comparable in size, suggesting the presence of significant populations of each conformer.

The lifetimes observed in the G283L CO flash-photolysis experiment are consistent with previous work on the G283V Rs aa3 enzyme, in which glycine was replaced by the larger valine residue [47]; Salomonsson et al. observed three lifetimes (40 μs, 400 μs, and 7 ms) upon recombination of CO to heme *a*3 in the G283V mutant. The 40 μs lifetime was attributed to CO recombination in the G283V enzyme [47]; this fast recombination lifetime led to the interpretation that the valine side chain obstructed the proposed ligand channel and effectively trapped CO within the heme pocket after photodissociation. We observed a similar 49 μs CO recombination process in the G283L Rs aa3, suggesting that this enzyme conformer effectively traps CO within the heme pocket after photolysis. However, we observed significant populations of additional G283L conformers, having CO recombination lifetimes of 487 μs and 9.4 ms, respectively. The G283L 9.4 ms process appears to be analogous to the 23 ms CO recombination in the wild-type enzyme, according to the similar shape of their respective *b*-spectra.
Interestingly, mutation of the analogous G232 residue to valine in *Tt ba* did not alter the kinetics of CO rebinding to heme $a_3$ [64]. The observed lifetime of CO recombination was 274 ms in the G232V $ba_3$ and 260 ms in the wild-type enzyme. The G232 residue in $Tt ba_3$ is located between two bulky amino acids, W229 and F228, which may restrict the movement of a side chain. Molecular dynamics simulations indicate that the replacement of G232 in $Tt ba_3$ with valine opens up other ligand exit/entry routes to the active site, enabling the protein to compensate for the larger valine residue [64].

**CO photodissociation and recombination in the W172Y/F282T Rs aa* mutant**

To study the roles of the W172 and F282 residues in modulating ligand access to the active site in *Rs aa*, we compared the CO rebinding in the wild-type enzyme and the W172Y/F232T mutant following photolysis of the respective reduced CO-bound enzyme. The TROA data for the wild-type enzyme were best fit with four lifetimes (Figure 13A) while three lifetimes were deemed adequate to fit the data obtained for the double mutant (Figure 17A). The first three lifetimes determined for the two enzymes are quite similar, and, importantly, the $b$-spectra associated with the first three lifetimes have the same shape. Figures 22 – 24 compare the first three $b$-spectra for the wild-type enzyme and the mutant with the respective spectra normalized for easier comparison; the $b$-spectra of the mutant have been red shifted ~5 nm to match those of the wild-type enzyme.
Figure 22. A comparison of the first $b$-spectra of the wild-type and W172Y/F282T mutant of $Rs$ $aa3$. The wild-type $b_1$-spectrum is shown in blue, and the W172Y/F282T $b_1$-spectrum is shown in green. The W172Y/F282T $b_1$-spectrum has been multiplied by a factor of 2.5 to normalize it to the wild-type $b_1$-spectrum.
**Figure 23.** A comparison of the second $b$-spectra of the wild-type and W172Y/F282T mutant of *Rs aa3*. The wild-type $b_2$-spectrum is shown in blue, and the W172Y/F282T $b_2$-spectrum is shown in green. The W172Y/F282T $b_2$-spectrum has been multiplied by a factor of 0.095 to normalize it to the wild-type $b_2$-spectrum.
Figure 24. A comparison of the third $b$-spectra of the wild-type and W172Y/F282T mutant of $Rs$ aa3. The wild-type $b_3$-spectrum is shown in blue, and the W172Y/F282T $b_3$-spectrum is shown in green. The W172Y/F282T $b_3$-spectrum has been multiplied by a factor of 0.4 to normalize it to the wild-type $b_3$-spectrum.
Based on the similarities between the first three \( b \)-spectra of the wild-type enzyme and the double mutant, including that of the \( \sim 44 \ \mu s \) \( b \)-spectrum associated with the CO recombination in the double mutant, we attribute the various processes to different CO conformers. The blue shift in the ground state optical absorption spectra as well as the TROA difference spectra of the mutant likely reflects the effect of structural changes caused by the mutations on the electronic structure of one or both hemes. In the wild-type enzyme and mutant, the CO conformers associated with the \( \sim 1 \ \mu s \) and \( \sim 300-500 \ \mu s \) processes are not heavily populated as reflected by the small amplitude \( b \)-spectra (Figure 13 and 17). However, the CO conformer corresponding to the \( \sim 50 \ \mu s \) process, is significantly more populated in the double mutant, and the structural changes resulting from the double mutation result in \( \sim 500 \) times faster rate of CO recombination compared to that in the wild-type enzyme. The most highly populated CO conformer in the wild-type \( Rs \ aa_3 \), associated with the 23 ms recombination, is not significantly populated in the double mutant.

The presence of CO conformers in \( Rs \ aa_3 \) is consistent with previously reported resonance Raman experiments on this enzyme [65] and FTIR studies of conformational substates of the fully reduced CO-bound enzyme [66]. Multiple CO conformers have also been reported for the bovine enzyme based on the infrared spectra of the CO-bound enzyme [25]. Hellvig and coworkers reported significant structural changes when the equivalent tryptophan residue in \( Paracoccus denitrificans \ aa_3 \), W(I-164), was replaced by a phenylalanine residue based on differences in the reduced-minus oxidized FTIR difference spectra between the
wild-type enzyme and the W164F mutant [67]. Ribacka et al. reported modest structural changes at the active site of *P. denitrificans* W164F as monitored by either optical or FTIR spectroscopy [68]. The latter group reported 4-times slower CO recombination in the *P. denitrificans* W164F mutant compared to the wild-type enzyme. Neither group addressed the effect of mutating simultaneously the tryptophan and phenylalanine residues on the CO recombination rate.

**Structural differences in the wild-type and W172Y/F282 Rs aa3 enzymes**

Classical molecular dynamics (MD) simulations of the wild-type and W172Y/F283T mutant of *Rs aa3* (all MD simulations and analyses, as well as the data interpretation, were accomplished by Dr. William McDonald, a postdoctoral scholar in our laboratory) have suggested changes in the hydrogen-bonding interactions of both enzymes. In the W172Y/F283T *Rs aa3* double mutant, the low-spin heme *a* maintains four H-bonds to water molecules and eight H-bonds to the protein. However, in the wild-type *Rs aa3*, heme *a* maintains six H-bonds to water molecules and 6.5 H-bonds to the protein. In these computational studies, a H-bond was observed between the heme *a* ring A propionate and the backbone N of Y483 in the W172Y/F283T *Rs aa3*; this H-bond interaction was not observed in the wild-type *Rs aa3*. These differences in H-bonding may alter the electrostatic environment of heme *a* in the W172Y/F282T *Rs aa3*.

Differences were also observed in the H-bond interactions of the high-spin heme *a3* of the wild-type and W172Y/F282T *Rs aa3* enzymes. In the W172Y/F282T
*Rs* aa₃ mutant, heme *a₃* maintains five H-bonds to the protein compared to 4.6 in the wild-type *Rs* aa₃. The double mutant also has 4.5 H-bonds to water compared to 7 H-bonds to water in the wild-type enzyme.

The classical MD simulations in our laboratory have also suggested that the W172Y/F282T mutation in *Rs* aa₃ causes a 3.8 Å displacement of the Mg²⁺ ion, which in turn displaces residues D412 and R481. The displacement of Mg²⁺ causes the side chain of R482 to shift 2.3 Å away from the heme *a₃* ring D propionate. The heme *a₃* ring D propionate rotates towards Cu_B and H-bonds with H334, a ligand of Cu_B. This movement of the ring D propionate and the H-bond to H334 may close off the ligand exit pathway from the binuclear center, providing a structural basis for the fast CO recombination in the W172Y/F282T *Rs* aa₃ enzyme.

**Conclusions**

The replacement of G283 by leucine introduces steric bulk in the proposed ligand channel of *Rs* aa₃, which may limit ligand access to the active site. The G283L mutation significantly reduces enzyme activity, and the air-oxidation of reduced G283L *Rs* aa₃ is several orders of magnitude slower compared to the wild-type enzyme. The ground state optical abruption spectrum of the as-isolated G283L *Rs* aa₃ suggests that the mutant enzyme (or a sub-population of the mutant enzyme) fails to complete a full redox cycle. CO flash-photolysis experiments on the G283L mutant also indicate the presence of a significant population of an enzyme conformer which recombines with CO ~49 μs after photodissociation of the heme *a₃*-CO complex. This
fast CO recombination lifetime is \(~500\) times faster than the major CO recombination process in the wild-type \(Rs\ a\alpha_3\); we interpret this result to suggest that CO is possibly trapped within the heme \(a_3\) pocket of this G283L enzyme conformer.

The G283 residue is immediately adjacent to F282, a bulky aromatic residue which, together with W172, defines a putative constriction point in the proposed ligand channel. The identity of the amino acid at position 283 in subunit I of \(Rs\ a\alpha_3\) is critical in facilitating ligand access to the active site. The strategic presence of glycine, the smallest amino acid, next to bulky aromatic residues may provide a small opening through which ligands can diffuse into or out of the heme \(a_3\) pocket.

The conserved G283 residue is located in an area near the active site where the proposed ligand channel overlaps with a putative gating region. It has been postulated that the mechanism of proton gating requires local structural rigidity near the PLS [47]. In their investigation of the G283V mutant of \(Rs\ a\alpha_3\), Salomonsson et al. suggested that protein motions in the vicinity of G283 are unable to compensate for the increased bulk of the valine side-chain [47]. In our CO recombination studies on the G283L \(Rs\ a\alpha_3\), we observe three enzyme conformers that are equally populated; one of these conformers, the 9 ms process, has a \(b\)-spectrum equivalent to that of the major conformer (23 ms) in the wild-type enzyme. Thus, mutation of G283 to leucine does not entirely trap the CO within the active site cavity after photolysis of the heme \(a_3\)-CO complex.

Our results also show that when W172 and F282—both of which are postulated to create a constriction point in the ligand channel of \(Rs\ a\alpha_3\)—are replaced
by the smaller tyrosine and threonine residues, respectively, present in Tt ba3, the rebinding of CO to heme a3 following photolysis of the reduced CO-bound mutant becomes ~500 times faster. Our results suggest that several CO conformers are present both in the wild-type Rs aa3 and the W172Y/F282T mutant but the contribution of each one varies between the two. Most importantly, the contribution of the CO conformer associated with the 45 µs process is very small in the wild-type enzyme, but because of structural changes at the active site arising from the double mutation, this CO conformer becomes the most prominent conformer in the W172Y/F282T mutant. Because the double mutation in Rs aa3 was constructed to “mimic” the ligand channel of Tt ba3, one might have expected the rate of CO recombination in the Rs aa3 W172Y/F282T mutant to be the same as that in the wild-type Tt ba3. However, this is clearly not the case; rather the CO recombination is ~6000 times faster in the Rs aa3 W172Y/F282T mutant than in the wild-type Tt ba3. Furthermore, when the tyrosine and threonine residues in the wild-type Tt ba3 were replaced by the tryptophan and phenylalanine residues present in the aa3 oxidases, the rate of CO recombination was the same. The lack of change in the rate of CO recombination between the wild-type Tt ba3 and the Y133W, T231F, and Y133W/T231F mutants is supported by the similarity between the crystal structures of the wild-type ba3 and the Y133W and Y133W/T231F mutants, reflected by an rmsd value of subunit I Cα atom of 0.18-0.25 Å [20]. The tryptophan side chain in the Tt ba3 Y133W and Y133W/T132F mutants extends into the ligand channel, resulting in 5-times slower rate of O2 and NO binding in these mutants compared to
the wild-type enzyme [20]; however, a key interaction with heme $a_3$ is maintained, with a hydrogen bond between the propionate of heme $a_3$ and the Y133 in the wild-type enzyme being replaced by a hydrogen bond between the propionate and the indole ring of the tryptophan in the mutants. In contrast, mutating the W172 and F282 residues in $Rs$ $aa_3$ to the tyrosine and threonine, respectively, causes structural changes at the active site of the *Rhodobacter* enzyme that effectively close off the exit of CO from the binuclear active site and/or prevent CO binding to CuB.
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Chapter 5

Time-Resolved Optical Absorption Studies of the Reactions of the Mixed-Valence *Escherichia coli bo*$_3$ Ubiquinol Oxidase and the Mixed-Valence *Rhodobacter sphaeroides aa*$_3$ Cytochrome *c* Oxidase with Dioxygen
Introduction

Heme copper oxidases (HCOs) are proton pumping enzymes of the aerobic respiratory chain in eukaryotes and many prokaryotes [1-3]. HCOs catalyze the highly exergonic reduction of molecular oxygen to water and couple this reaction with the translocation of protons across the inner mitochondrial membrane in mammals or the plasma membrane in bacteria [4]. The resulting transmembrane electrochemical proton gradient serves as a source of potential energy which may be utilized to drive energetically unfavorable processes or reactions within the cell. HCOs may be classified according to the identity of the electron donor they oxidize. For example, cytochrome c oxidases (CcOs) catalyze the oxidation of cytochrome c, a water soluble protein, whereas ubiquinol oxidases catalyze the oxidation of the ubiquinol, a membrane soluble molecule.

The critical role of HCOs in bioenergetics has sparked considerable interest in elucidating the chemistry of redox-driven energy transduction in HCOs. Of all the HCOs, bovine heart CcO has been the most extensively investigated. The reaction mechanism and kinetics of dioxygen reduction in bovine heart CcO has been studied by time-resolved absorbance spectroscopy [5, 6] in combination with the CO flow-flash technique [7]. The reaction mechanism has generally been interpreted in terms of a unidirectional sequential scheme involving several intermediates defined by the oxidation/protonation states of the redox-active metal centers (Figure 1) [5, 6, 8].

Bovine heart CcO is a 13 subunit protein complex coded for by mitochondrial and nuclear DNA [9]. The relatively complex physical structure of bovine heart CcO,
Figure 1. Conventional unidirectional sequential reaction mechanism for dioxygen reduction in the fully reduced bovine heart CcO. Image from reference [6].
as well as the dual genetic origin of its protein subunits, has hampered the elucidation of structure-function relationships in the mammalian enzyme. Two bacterial oxidases that are often used as models for mammalian CcO are the \textit{aa3}-type CcO from \textit{Rhodobacter sphaeroides} (\textit{Rs aa3}) [10, 11], and the \textit{bo3}-type ubiquinol oxidase from \textit{Escherichia coli} (\textit{Ec bo3}) [12, 13]. The \textit{Rs aa3} and \textit{Ec bo3} enzymes are similar to bovine heart CcO in terms of their overall function and catalytic subunit structure [11, 13, 14]. The bacterial and mammalian oxidases reduce dioxygen to water using similar redox-active cofactors, and the redox chemistry in each enzyme takes place at a heme-copper binuclear active site.

The \textit{Rs aa3} and \textit{Ec bo3} enzymes, which belong to the A-family of HCOs [2, 3], share several common traits. Both catalyze the four electron reduction of dioxygen to water and translocate four protons across the cytoplasmic membrane. Both oxidases have similar structural architectures, and, indeed, their structures are superimposeable [11, 13]. The \textit{Rs aa3} and \textit{Ec bo3} are comprised of four subunits and are amenable to genetic manipulation, making them useful systems for protein expression. Electron transfer from cytochrome \textit{c} drives proton pumping in \textit{Rs aa3}, while \textit{Ec bo3} couples the oxidation of ubiquinol-8 (referring to 8 isoprenoid units in the molecule's hydrophobic tail) with proton pumping. Three redox-active metal centers are found within subunit I in each oxidase: a low-spin heme Fe (heme \textit{a} in \textit{Rs aa3} and heme \textit{b} in \textit{Ec bo3}), and a binuclear active site comprised of CuB and a high-spin heme Fe (heme \textit{a3} in \textit{Rs aa3} and heme \textit{o3} in \textit{Ec bo3}), where the dioxygen binding and reduction take
place [11, 13]. However, Ec bo3 does not contain the fourth redox center present in the Rs aa3 oxidase—the dinuclear CuA site located within subunit II of Rs aa3.

Single-wavelength transient absorption spectroscopy and resonance Raman spectroscopy have been used to explore the mechanism of dioxygen reduction in the fully reduced Rs aa3 and Ec bo3 [15-21]. Until recently, the reaction mechanism of dioxygen reduction in the fully reduced Rs aa3 and E. coli bo3 enzymes was thought to be analogous to the unidirectional sequential scheme proposed for the fully reduced bovine heart CcO: R → A → Pr → F → O. Single-wavelength CO flow-flash studies of the reaction between the fully reduced E. coli bo3 and O2 reported three kinetic phases attributed to the transition of R to P, P to F, and F to O [20]; however, compound A was undetected. Multi-wavelength CO flow-flash experiments in our laboratory have suggested that dioxygen reduction in the fully reduced Rs aa3 occurs with the sequential formation of intermediates R, compound A, F, and O; compound A was observed to decay directly to F without the detectable formation of Pr in between [22]. Recent multi-wavelength TROA studies in our laboratory on the reaction of O2 with the fully reduced Ec bo3 indicated that compound A decays to a mixture of Pr (~30%) and F (~70%), followed by F [23]; the re-reduction of heme b—presumably through electron transfer from ubiquinol to form semiquinone—was observed to have a lifetime of ~30 µs, significantly faster than the 700 µs lifetime previous reported for this process [20].

The mechanisms of dioxygen reduction in the fully reduced Rs aa3 and Ec bo3 enzymes proposed by our laboratory share a common feature: the primary
intermediate following splitting of the O–O bond is the oxo-ferryl intermediate F and
not Pr. Previous TROA studies in our laboratory have indicated that the so-called Pr
intermediate in the bovine enzyme is not a pure P form; rather, it is better modeled by
a mixture of compound A, P and F [24]. In the reaction between O₂ and the fully
reduced Thermus thermophilus ba₃, compound A decays to P; during the subsequent
electron transfer between Cuₐ and heme a, heme a₃ maintains the spectral
characteristics of the P intermediate rather than being converted to the F form [25].

In the presence of CO, oxidized HCOs may be converted to the two-electron
reduced mixed-valence CO-bound species, in which the binuclear center is reduced,
and the other redox-active metal centers are oxidized [5]. In the mixed-valence CO-
bound (MVCO) form of Rs aa₃, CO is bound to the ferrous heme a₃ iron (Fe²⁺), and
Cuₜ exists in the +1 state, whereas Cuₐ and the heme a iron are oxidized (Cuₐ⁺² and
Fe³⁺). The MVCO Ec bo₃ enzyme contains CO-bound ferrous heme o₃, cuprous Cuₜ,
and oxidized heme b. Previous transient absorption studies reported the formation of
Pₘ after photolysis of the MVCO Rs aa₃ enzyme in the presence of O₂ [26]. When
the oxidized Ec bo₃ was mixed with hydrogen peroxide and the MVCO bo₃ was
photolyzed in the presence of O₂, an intermediate with an absorbance maximum at
582 nm and a shoulder near 550 nm was observed, followed by the formation of an
intermediate absorbing at 557 nm [27]; the 582 nm species was identified as Pₘ and
the 557 nm species was attributed to the oxyferryl F intermediate. In a different study,
the reaction of the oxidized bo₃ with H₂O₂ produced an intermediate with a peak at
550 nm and a shoulder at 582 nm [28].
In the present study, we investigated the reactions between the mixed-valence Rs aa₃ and Ec bo₃ with O₂ in the presence of CO using multi-wavelength time-resolved optical absorption spectroscopy, in combination with the CO flow-flash technique. Our results show that in the mixed-valence Rs aa₃ enzyme (in the presence of CO), compound A is converted to the Pₘ form, whereas in the mixed-valence Ec bo₃ (in the presence of CO), compound A is converted to a mixture of intermediates Pₘ and F.

Materials and Methods

Professor Robert Gennis, University of Illinois at Urbana-Champaign, generously provided the wild-type cytochrome bo₃ ubiquinol oxidase from E. coli. The wild-type fusion R. sphaeroides strain was graciously provided by Professor Shelagh Ferguson-Miller, Michigan State University.

All chemicals used were of the highest purity reagent grade. The detergent DM was obtained from Anatrace. Ni-NTA resin was obtained from Qiagen. Standard grade (99.9%) carbon monoxide was purchased from Praxair. All other chemicals were from Fisher Biosciences and Sigma-Aldrich unless otherwise noted.

The wild-type Ec bo₃ and the wild-type fusion Rs aa₃

The wild-type fusion Rs aa₃ was previously described in the Materials and Methods section of Chapter 2; details pertaining to bacterial growth and protein purification of the wild-type fusion Rs aa₃ may also be found in Chapter 2.
A plasmid containing the cyoABCDE operon, which encodes the entire cytochrome bo$_3$ enzyme complex, was engineered with a hexa-His tag at the C-terminal end of subunit II [29]. The plasmid was cloned into the expression vector pET-17b (Novagen) under the control of a T7 RNA polymerase promoter [30]. Subunits I, II, III, and IV of Ec bo$_3$ are encoded by the cyoB, cyoA, cyoC, and cyoD genes, respectively [31, 32]; cyoE codes for heme O synthase, which supplies heme O necessary for the functional assembly of the heme o$_3$-Cu$_{B}$ binuclear center of the enzyme [33]. The recombinant pET-17b vector was transformed into E. coli BL21(DE3) cells, which contain a gene for T7 RNA polymerase controlled by the lacUV5 promoter [34]. The addition of IPTG (isopropyl-β-d-thiogalactopyranoside) to a bacterial culture of transformed BL21(DE3) induces expression of T7 polymerase, which binds specifically with the T7 promoter on the pET-17b vector; this, in turn, initiates the expression of Ec bo$_3$ encoded by plasmid pET-17b. The pET-17b and BL21(DE3) system was used due to its high yield of Ec bo$_3$ derived from bacterial growth in minimal media. Upon IPTG induction of T7 RNA polymerase, the population of mRNAs (encoding the desired gene product) increases to such a degree that the cell essentially becomes dedicated to producing the desired protein.

The transformed E. coli strain BL21(DE3) was grown under aerobic conditions in M63 minimal media at 37°C, with constant shaking (~200 rpm). Bacteria were grown until the OD$_{600}$ reached ~0.6, whereupon the over-expression of the wild-type Ec bo$_3$ was induced by the addition of IPTG (0.5 mM, final concentration); the bacteria were incubated at 37°C for an additional ~4–5 hours. The
wild-type Ec bo3 was isolated and purified via Ni-NTA chromatography as previously described [30]. The final enzyme was solubilized in 50 mM K-phosphate (pH 7.5) chloride-free buffer containing 0.05% DDM detergent (Affymetrix). Heme analysis and the ubiquinone content in the purified enzyme preparations were determined as previously reported [29, 35].

Ground state UV-visible absorption spectra of the Ec bo3 and Rs aa3 enzymes in various oxidation states were recorded on an 8453 HP spectrophotometer and compared with previously published spectra. The Ec bo3 concentration was determined using an extinction coefficient of $\Delta\varepsilon_{560-580} = 24 \text{ mM}^{-1}\text{cm}^{-1}$ for the reduced-minus-oxidized difference spectrum [36]. The Rs aa3 concentration was determined using an extinction coefficient of $\Delta\varepsilon_{606-650} = 41 \text{ mM}^{-1}\text{cm}^{-1}$ for the reduced spectrum [10].

*Preparation of the mixed-valence Rs aa3 and Ec bo3 enzymes*

The mixed-valance CO-bound enzymes were prepared in the following manner: the enzyme sample was diluted in 100 mM HEPES buffer (pH 7.4), supplemented with 0.1% DDM. The enzyme was deoxygenated by degassing the sample on a vacuum line, followed by flushing with inert nitrogen gas; this process was performed a minimum of three times. The enzyme sample was then exposed to CO gas at room temperature for 1–3 hours or overnight, with occasional agitation. Ferricyanide was added (final concentration ~50 μM) when the samples were "over-reduced"; addition of $[\text{Fe(CN)}_6]^{3-}$ reoxidizes the low-spin heme, while keeping the
high-spin heme reduced and CO-bound. The sample was incubated at room temperature until the enzyme reached the mixed-valence CO-bound state, as reflected by the absorption spectra.

**CO flash-photolysis of the MVCO Rs aa₃ and Ec bo₃**

CO recombination following photolysis of the MVCO Rs aa₃ or Ec bo₃ was investigated using TROA spectroscopy. The flash-photolysis experiments on the MVCO enzymes were performed at room temperature in a deoxygenated quartz cuvette. Photodissociation of the enzyme-CO complex was achieved using a 532 nm pulse from a Q-switched DCR-11 Nd:YAG laser (~7 ns full width at half maximum). TROA spectra (post- minus pre-photolysis) were recorded in the 350 – 700 nm region, at logarithmically spaced time points between 100 ns to 500 ms for both enzymes. The TROA difference spectra were analyzed by SVD and global exponential fitting (refer to Appendix I for further details regarding the SVD and global exponential fit analysis) using Matlab (Mathworks).

**CO flow-flash experiments of the MVCO Rs aa₃ or Ec bo₃ in the presence of O₂**

The reaction of O₂ with the mixed-valence Rs aa₃ or *E. coli* bo₃ was investigated using the CO flow-flash method in which the respective mixed-valence CO-bound enzyme (MVCO) was mixed with O₂, followed by photolysis of CO from heme a₃ or heme o₃ with a 532 nm laser pulse. TROA difference spectra were recorded using a charge coupled device (CCD) camera at logarithmic delay times.
after CO photolysis (1 µs to 2 ms for both the *E. coli* bo*₃* and *Rs* aa*₃*). The spectra were analyzed by SVD and global exponential fitting, followed by mechanistic analysis and the extraction of the intermediate spectra, as previously described [24, 37]. A good agreement between the experimental intermediate spectra and model spectra of the proposed intermediates was used as criteria for the validity of the proposed mechanism. The mixed-valence *Rs* aa*₃* intermediate spectra were modeled against the spectra of intermediates generated during the reaction between O₂ and the mixed-valence bovine heart enzyme. The mixed-valence *E. coli* bo*₃* intermediate spectra were compared to the frequency-shifted bovine aa*₃* intermediate spectra from CO/O₂ flow-flash experiments on this enzyme [23].

**Results**

*Ground state absorption spectra of the wild-type Ec bo*₃* and Rs aa*₃*

Figure 2A shows the ground state optical absorption spectra of the oxidized, fully reduced, and fully reduced CO-bound (FRCO) forms of the wild-type *Ec bo₃*. The oxidized *Ec bo₃*, which contains ferric heme *b* and heme *o₃*, exhibits an absorbance maximum at ~409 nm. The reduced enzyme (which contains ferrous heme *b* and heme *o₃*) exhibits an intense peak at 428 nm and two additional absorption maxima at 532 nm and 563 nm. The spectrum of the FRCO enzyme contains absorption maxima at 532 nm and 562 nm, as well as an intense Soret peak at 418 nm with a shoulder at ~428 nm. The absorbance contribution of Cuₐ is unknown.
Figure 2B shows the oxidized-minus-reduced and reduced-minus-FRCO difference spectra for the *Ec bo*$_3$ enzyme. The oxidized-minus-reduced difference spectrum contains absorption minima at 428 nm, 532 nm, and 562 nm. The reduced-minus-FRCO difference spectrum exhibits an absorbance minimum at ~416 nm and an absorbance maximum at ~430 nm.

Figure 3A shows the ground state optical absorption spectra of the oxidized, fully reduced, and FRCO forms of the wild-type fusion *Rs aa*$_3$. The oxidized *Rs aa*$_3$ exhibits absorbance maxima at ~422 nm and ~602 nm. The reduced enzyme exhibits absorbance maxima at ~445 nm and ~606 nm. The spectrum of the FRCO enzyme contains absorption maxima at ~435 nm and ~590 nm.

Figure 3B shows the oxidized-minus-reduced and reduced-minus-FRCO difference spectra for the *Rs aa*$_3$ enzyme. The oxidized-minus-reduced difference spectrum contains absorption minima at ~446 nm and ~606 nm. The reduced-minus-FRCO difference spectrum exhibits an absorbance minimum at ~431 nm and an absorbance maximum at ~446 nm.

The ground state optical absorption spectra of the oxidized and mixed-valence CO-bound (MVCO) *Ec bo*$_3$ are shown in Figure 4A. The MVCO enzyme displays an intense peak at ~416 nm. The oxidized-minus-MVCO difference spectrum, shown in Figure 4B, exhibits an absorption minimum at 418 nm.

Figure 5A shows the ground state absorption spectra of the oxidized and MVCO *Rs aa*$_3$. The MVCO spectrum of the *Rs aa*$_3$ enzyme exhibits an absorption
**Figure 2.** UV-visible ground state and difference spectra of the oxidized, reduced, and FRCO forms of Ec bo₃. Panel A: the oxidized Ec bo₃ (blue); the fully reduced Ec bo₃ (green); the FRCO Ec bo₃ (red). Panel B: The oxidized-minus-reduced (blue) and reduced-minus-FRCO (green) difference spectra for the Ec bo₃ enzyme.
Figure 3. UV-visible ground state and difference spectra of the oxidized, reduced, and FRCO forms of the wild-type fusion Rs aa₃. Panel A: the oxidized Rs aa₃ (blue); the fully reduced Rs aa₃ (green); the FRCO Rs aa₃ (red). Panel B: The oxidized-minus-reduced (blue) and reduced-minus-FRCO (green) difference spectra for the Rs aa₃ enzyme.
Figure 4. UV-visible ground state and difference spectra of the oxidized and MVCO forms of *Ec bo3*. Panel A: the oxidized *Ec bo3* (blue); the MVCO *Ec bo3* (green).

Panel B: The oxidized-minus-MVCO difference spectrum (blue) of the *Ec bo3* enzyme.
Figure 5. UV-visible ground state and difference spectra of the oxidized and MVCO forms of the wild-type fusion *Rs aa*3. Panel A: the oxidized *Rs aa*3 (blue); the MVCO *Rs aa*3 (green). Panel B: The oxidized-minus-MVCO difference spectrum (blue) for the *Rs aa*3 enzyme.
maximum at ~415 nm. The oxidized-minus-MVCO difference spectrum, shown in Figure 5B, contains an absorption minimum at ~433 nm.

**CO recombination following photolysis of the MVCO Ec bo3 and Rs aa3**

In the CO-bound mixed-valence Ec bo3 or Rs aa3, the two electrons reside at the binuclear center, while the low-spin heme (and CuA, in the case of Rs aa3) remains oxidized. The intramolecular electron transfer dynamics and CO recombination rate in Ec bo3 or Rs aa3 may be investigated by photodissociating the mixed-valence enzyme-CO complex and monitoring the spectral changes over time.

Figure 6 shows the TROA difference spectra (post- minus pre-photolysis) recorded during the CO flash-photolysis experiment of the MVCO Rs aa3 enzyme. SVD analysis and global exponential fitting of the spectral data revealed four apparent lifetimes: 300 ns, 2 μs, 37 μs, 67 ms, and 250 ms. The b-spectra associated with the apparent lifetimes are shown in Figure 7A, and the residuals resulting from the five-exponential fit are shown in Figure 7B. The b-spectra are in good agreement with our previously published b-spectra of the MVCO Rs aa3 [38]. The 67 ms (b4) and 250 ms (b5) processes are attributed to CO recombination. The spectra of b4 and b5 have the same spectral shape; however, the b5-spectrum has a larger amplitude than the b4-spectrum. This suggests the presence of two different enzyme conformers. The conformer which exhibits the 250 ms CO recombination rate is in higher abundance than the enzyme conformer which corresponds to the slower rate. These findings are
Figure 6. TROA difference spectra (post- minus pre-photolysis) recorded in the Soret and visible regions following photolysis of the MVCO Rs aa₃ enzyme.
Figure 7. The $b$-spectra (Panel A) and residuals (Panel B) resulting from a five-exponential fit of the SVD-filtered TROA data recorded following photolysis of the MVCO Rs aas.
in good agreement with previously published mixed-valence Rs aa3 CO recombination experiments, which reported 68 ms and 240 ms CO recombination rates [38]. The extent of electron back-flow from heme $a_3^{2+}$ to heme $a^{3+}$, determined as previously described in [38], was ~70% (data not shown), which is in excellent agreement with the ~75% electron back-flow observed in previous published studies from our lab [38].

Figure 8 shows the TROA difference spectra (post- minus pre-photolysis) recorded in the Soret and visible regions following photolysis of the MVCO Ec bo3 enzyme. The SVD-filtered spectral data were adequately fitted with three exponentials; the apparent lifetimes were: 8 μs, 19 ms, and 500 ms. Figure 9A shows the b-spectra associated with the apparent rates, and Figure 9B shows the residuals resulting from the three-exponential fit. The 19 ms is attributed to CO recombination. The extent of electron back-flow in the mixed-valence Ec bo3 was previously estimated to be ~8% [39].

Conventional CO flow-flash experiments following photolysis of the MVCO Ec bo3 or Rs aa3 in the presence of $O_2$

Figure 10 shows the TROA difference spectra (post- minus pre-photolysis) collected during the reaction between dioxygen and the mixed-valence Ec bo3. SVD analysis and global exponential fitting of the time-resolved difference spectra revealed two lifetimes, 28 μs and 142 μs. Figure 11A shows the b-spectra and
Figure 8. TROA difference spectra (post- minus pre-photolysis) recorded in the Soret and visible regions following photolysis of the MVCO Ec bo$_3$ enzyme.
**Figure 9.** The $b$-spectra (Panel A) and residuals (Panel B) resulting from a three-exponential fit of the SVD-filtered TROA data recorded following photolysis of the MVCO Ec bo3.
Figure 10. TROA difference spectra (post- minus pre-photolysis) recorded in the Soret and visible regions following photolysis of the MVCO Ec bo3 enzyme in the presence of dioxygen.
Figure 11. The $b$-spectra (Panel A) and residuals (Panel B) resulting from a two-exponential fit of the SVD-filtered TROA data recorded following photolysis of the MVCO Ec bo$_3$ in the presence of dioxygen.
residuals corresponding to the two-exponential fit. The two-exponential fit is in good agreement with the experimental data as indicated by the presence of only random noise in the residuals (Figure 11B). The nature of the intermediates generated during the reaction of dioxygen with mixed-valence Ec bo3 in the presence of CO will be discussed in detail below.

Figure 12 shows the TROA difference spectra (post- minus pre-photolysis) acquired during the reaction between dioxygen and the mixed-valence Rs aa3. Global exponential fitting of the SVD-filtered TROA data resolved three lifetimes for Rs aa3: 2 µs, 31 µs and 470 µs. The b-spectra and residuals corresponding to the three-exponential fit are shown in Figure 13A and 13B, respectively. The residuals show a good correlation between the experimental data and the three-exponential fit, with only random noise in each trace. The spectral intermediates generated during the reaction of O2 with the mixed-valence Rs aa3 in the presence of CO will be discussed in detail below.

Discussion

Spectral intermediates generated following photolysis of the MVCO Ec bo3 enzyme in the presence of O2

Recent CO flow-flash studies on the reaction between dioxygen and the fully reduced Ec bo3 enzyme in our laboratory indicated that compound A decays to intermediate F via the formation of a transient mixture of PR and F [23]. Upon breakage of the dioxygen double bond, compound A was observed to decay with a
Figure 12. TROA difference spectra (post- minus pre-photolysis) recorded in the Soret and visible regions following photolysis of the MVCO Rs aa3 enzyme in the presence of dioxygen.
**Figure 13.** The $b$-spectra (Panel A) and residuals (Panel B) resulting from a three-exponential fit of the SVD-filtered TROA data recorded following photolysis of the MVCO *Rs aa*$_3$ in the presence of dioxygen.
lifetime of ~22 μs, forming a ~30/70 mixture of \textbf{P}\textsubscript{R}/\textbf{F}; this mixture, in turn, decayed with a lifetime of ~30 μs to \textbf{F}. Whether compound \textbf{A} in the mixed-valence \textit{bo}\textsubscript{3} decays to the \textbf{P} or \textbf{F} state is unclear. To provide insight into the identities of the intermediates generated during the reaction of the mixed-valence \textit{Ec} \textit{bo}\textsubscript{3} with dioxygen, we used the CO flow-flash method, in combination with multi-wavelength TROA spectroscopy, to monitor the reaction following photodissociation of the MVCO enzyme in the presence of \textit{O}_2. Two exponentials were sufficient to adequately fit the SVD-filtered TROA data, and a two-step unidirectional sequential mechanism, shown in Scheme 1, was used to extract the spectra of the intermediates (Figure 14).

To test the validity of the proposed two-step mechanism, the experimentally derived \textit{Ec} \textit{bo}\textsubscript{3} intermediate spectra were compared on a side-by-side basis with model spectra of the proposed intermediates. In modeling the \textit{Ec} \textit{bo}\textsubscript{3} intermediate spectra, we used the frequency-shifted spectra of intermediates \textbf{A}, \textbf{P}\textsubscript{M}, and \textbf{F} obtained from CO-O\textsubscript{2} flow-flash experiments on the mixed-valence bovine heart \textit{aa}\textsubscript{3} in the presence of dioxygen. Because the bovine absorption spectra are red shifted by 25 nm in the visible region and 13 nm in the Soret region (relative to heme \textit{o}\textsubscript{3}), the spectra of the bovine \textit{aa}\textsubscript{3} and \textit{Ec} \textit{bo}\textsubscript{3} cannot be directly compared to each other. However, during our recent studies of the reaction of the fully reduced \textit{Ec} \textit{bo}\textsubscript{3} with \textit{O}_2, we showed that shifting the relevant spectra of the bovine heme \textit{a}\textsubscript{3} on the energy or frequency scale resulted in good agreement between the intermediate spectra of heme \textit{o}\textsubscript{3} and heme \textit{a}\textsubscript{3} [23]. The present study employs this energy scale approach to
Scheme 1. A unidirectional sequential scheme showing three intermediates during the reaction of dioxygen with the mixed-valence Ec bo₃.
Figure 14. The intermediate spectra extracted from the TROA difference spectra generated following photodissociation of the MVCO Ec bo3 in the presence of dioxygen. **Intermediate 1** is shown in blue, **Intermediate 2** in green, and **Intermediate 3** in red.
compare the spectral intermediates generated during the reaction of the mixed-valence Ec bo3 or bovine aa3 with O2.

Based on the proposed mechanism in Scheme 1, the experimental spectrum of Intermediate 1 (referenced versus the MVCO enzyme) was extracted (Figure 15, blue curve). Intermediate 1 represents the partially reduced intermediate R_M, in which heme o3 and CuB are reduced and heme b is oxidized. Figure 15 compares the spectrum of Ec bo3 Intermediate 1 (blue curve) with the frequency-shifted spectrum of Rm (green curve) generated during the reaction of the mixed-valence bovine aa3 with O2; both intermediate spectra are referenced versus the MVCO forms of the respective enzyme. The bovine and Ec bo3 spectra have been normalized based upon to equivalent enzyme concentrations. Because the low-spin hemes are oxidized in the mixed-valence enzymes and the spectra are referenced versus the MVCO form, any spectral contribution due to the low-spin heme a or heme b is effectively subtracted out. As shown in Figure 15, the spectral shapes of Ec bo3 Intermediate 1 and bovine R_m are consistent with each other. However, as noted previously [23], there is a difference in the amplitudes of the absorption minima at ~415 nm, with the amplitude of the bovine R_m spectrum being larger than the Ec bo3 Intermediate 1; this difference in amplitude is due to the extinction coefficient of the CO-bound heme o3^{2+} in Ec bo3 being larger than that of the heme a3 in the CO-bound bovine enzyme, as discussed in our previous work. [23].

Figure 16 compares the experimental intermediate spectrum of Ec bo3 Intermediate 2 (blue curve), determined based upon the proposed mechanism in
**Figure 15.** A comparison of the spectrum of *Ec bo*$_{3}$ Intermediate 1, generated during the reaction between dioxygen and the mixed-valence *Ec bo*$_{3}$, and the frequency-shifted spectrum of bovine R$_{M}$, generated during the reaction between dioxygen and the mixed-valence bovine heart aa$_{3}$. The spectrum of *Ec bo*$_{3}$ Intermediate 1 is shown in blue. The spectrum of bovine aa$_{3}$ R$_{M}$ is shown in green. The intermediate spectra are referenced versus the respective MVCO enzyme.
Figure 16. A comparison of the spectrum of *Ec bo*$_3$ Intermediate 2, generated during the reaction between dioxygen and the mixed-valence *Ec bo*$_3$ (blue curve), and the frequency-shifted spectrum of bovine *Am*, generated during the reaction between dioxygen and the mixed-valence bovine heart *aa*$_3$ (green curve). The intermediate spectra are referenced versus the respective MVCO enzyme.
Scheme 1, with the frequency-shifted spectrum of compound A_M (green curve), generated during the reaction of the mixed-valence bovine aa_3 with O_2. The extent of electron back-flow following CO photolysis of the MVCO Ec bo_3 enzyme is small (~8%) [39], and therefore it is not corrected for in the model spectrum. The shapes of the Ec bo_3 Intermediate 2 spectrum and the frequency-shifted bovine A_M spectrum are in good agreement in the Soret region; however, there is some discrepancy in the visible region, which suggests some differences in the size of the energy gap between the electronic states of heme a_3 and heme o_3 in the Soret and visible regions [23]. An O_2 binding lifetime of 28 µs was observed for the reaction of the mixed-valence Ec bo_3 with dioxygen, which corresponds to a second-order rate constant of ~5.9×10^{-7} M^{-1}s^{-1}; this value is similar to the ~3.9×10^{-7} M^{-1}s^{-1} previously determined for O_2 binding in the fully reduced Ec bo_3 [23].

Figure 17 compares the experimental spectrum of Ec bo_3 Intermediate 3 (blue curve), determined based upon the proposed mechanism in Scheme 1, with the spectrum of the frequency-shifted bovine P_M (green curve), generated during the reaction of the mixed-valence bovine aa_3 with O_2; the frequency-shifted spectrum of bovine F (red curve), generated during dioxygen reduction in the fully reduced bovine enzyme, is also shown for comparison. In the visible region, it is clear that the spectrum of Ec bo_3 Intermediate 3 does not match the frequency-shifted spectrum of bovine P_M. The bovine heart P_M absorbs strongly at 607 nm, adjusted to 580 nm in the frequency-shifted spectrum, whereas the amplitude of the 580 nm peak in the Ec bo_3 Intermediate 3 is much smaller. The experimental spectrum of Ec bo_3
Figure 17. A comparison of the spectrum of *Ec bo*3 Intermediate 3, generated during the reaction between dioxygen and the mixed-valence *Ec bo*, and the frequency-shifted spectra of bovine Pm and F. The spectrum of *Ec bo*3 Intermediate 3 is shown in blue. The intermediate spectrum of bovine Pm (green curve) was generated during the reaction between dioxygen and the mixed-valence bovine heart aa3, and is referenced versus the MVCO bovine enzyme. The intermediate spectrum of bovine F (red curve) was generated during the reaction between dioxygen and the fully reduced bovine heart aa3, and is referenced versus the FRCO bovine enzyme.
Intermediate 3 corresponds reasonably well with the frequency-shifted spectrum of bovine F, although the presence of a fraction of Pm cannot be ruled out. Indeed, as shown in Figure 18, the spectrum of Ec bo3 Intermediate 3 is best modeled by a 40/60 mixture of bovine P and F model spectra. Thus in the reaction of the mixed-valence Ec bo3 with O2, the primary intermediate following the decay of compound A is the oxyferryl F state. Based on the above analysis, we propose the mechanism in Figure 19 for the reaction of the mixed-valence Ec bo3 with O2.

Spectral intermediates generated following photolysis of the MVCO Rs aa3 enzyme in the presence of O2

Recent TROA experiments in our laboratory on the reaction of the fully reduced Rs aa3 with O2 have suggested that compound A (A_R) decays directly to F without detectable formation of intermediate P [22]. In light of these results, we examined the nature of the final intermediate in the reaction of the mixed-valence Rs aa3 with O2. Three exponentials—yielding lifetimes of 2 µs, 31 µs and 470 µs—were sufficient to fit the SVD-filtered TROA data acquired during the reaction of the mixed-valence Rs aa3 with O2. The backflow of electrons from heme a3²⁺ to heme a³⁺ in the mixed-valence Rs aa3 is significant; previous studies have estimated ~75% electron back-flow [38]. Thus, electron back-flow must be taken into consideration when determining the intermediate spectra in the reaction of the mixed-valence Rs aa3 with O2. A four-intermediate mechanism which takes into account electron
Figure 18. A comparison of the spectrum of *Ec bo*$_3$ Intermediate 3, generated during the reaction between dioxygen and the mixed-valence *Ec bo*$_3$, and the frequency-shifted spectrum representing a 40/60 mixture of bovine Pm and F model spectra. The spectrum of *Ec bo*$_3$ Intermediate 3 (blue curve) is referenced versus the oxidized *bo*$_3$ enzyme. The bovine model spectrum (green curve) is referenced versus the oxidized bovine enzyme.
Figure 19. Proposed mechanism for the reaction of the mixed-valence $Ec bo_3$ with $O_2$. 

\[ R_M \xrightarrow{28 \mu s} A_M \xrightarrow{142 \mu s} P_M / F \]

- $O_3^{2+} + Cu_B^{3+}$
- $O_3^{2+} - O_2$
- $Cu_B^{3+} + H^+$
- $(o_3 = O)^{2+}$
- $HO-Cu_B^{2+} / H_2O-Cu_B^{2+}$
- $40\% / 60\%$
back-flow during the mixed-valence *Rs aa3* CO-O₂ flow-flash experiment is proposed in Scheme 2. Based upon this mechanism, the spectra of the intermediates were extracted (Figure 20).

Figure 21 shows the extracted experimental spectrum of *Rs aa3* Intermediate 1 (blue curve), which is well-modeled by the spectrum of bovine *R₉M* (green curve), generated during the reaction of the mixed-valence bovine *aa3* with the dioxygen.

Figure 22 compares the spectrum of *Rs aa3* Intermediate 3 (blue curve) in Scheme 2 with the spectrum of bovine *A₉m* (blue curve). The spectral shapes are in good agreement, with some discrepancy in the visible region, possibly due to slight differences in the energies of the heme *a₃* electronic states between the *Rs aa3* and bovine enzymes; this is consistent with the visible ground state spectra of *Rs aa3* being slightly red shifted compared to the bovine enzyme. We observed a lifetime of 31 µs for O₂ binding in the mixed-valence *Rs aa3*, which corresponds to a second-order rate constant of ∼5.4×10⁻⁷ M⁻¹s⁻¹. Previous studies on dioxygen reduction in the fully reduced *Rs aa3* reported a second-order rate constant of ∼9.1×10⁻⁷ M⁻¹s⁻¹ for O₂ binding.

The experimental spectrum of *Rs aa3* Intermediate 4 in Scheme 2 for the mixed-valence enzyme (Figure 23, blue curve), the intermediate formed upon the decay of *A₉m*, is in excellent agreement with the spectrum of bovine *P₉m* (green curve). Figure 24 shows a one-on-one comparison of the spectrum of the intermediate formed upon the decay of *A₉R* in the fully reduced *Rs aa3* with the spectrum of bovine *F*, generated during dioxygen reduction in the fully reduced bovine *aa3*. Thus, although
Scheme 2. A four-intermediate unidirectional sequential scheme taking into consideration electron back-flow during the reaction of dioxygen with the mixed-valence $Rs\ aas$. 
**Figure 20.** The intermediate spectra extracted from the TROA difference spectra generated following photodissociation of the MVCO *Rs aa3* in the presence of dioxygen. **Intermediate 1** is shown in blue, **Intermediate 2** in green, **Intermediate 3** in red, and **Intermediate 4** in cyan.
Figure 21. A comparison of the spectrum of *Rs aa*₃ Intermediate 1 in Scheme 2, generated during the reaction between dioxygen and the mixed-valence *Rs aa*₃, and the spectrum of bovine *R*M, generated during the reaction between dioxygen and the mixed-valence bovine heart *aa*₃. The spectrum of *Rs aa*₃ Intermediate 1 is shown in blue. The spectrum of the bovine *aa*₃ *R*M is shown in green. The intermediate spectra are referenced versus the respective MVCO enzyme.
Figure 22. A comparison of the spectrum of *Rs* *aa*₃ Intermediate 3 in Scheme 2, generated during the reaction between dioxygen and the mixed-valence *Rs* *aa*₃, and the spectrum of bovine *A*M, generated during the reaction between dioxygen and the mixed-valence bovine heart *aa*₃. The spectrum of *Rs* *aa*₃ Intermediate 3 is shown in blue. The spectrum of the bovine *aa*₃ *A*M is shown in green. The intermediate spectra are referenced versus the respective MVCO enzyme.
Figure 23. A comparison of the spectrum of *Rs aa*$_3$ Intermediate 4 in Scheme 2, generated during the reaction between dioxygen and the mixed-valence *Rs aa*$_3$, and the spectrum of bovine P$_{M}$, generated during the reaction between dioxygen and the mixed-valence bovine heart *aa*$_3$. The spectrum of *Rs aa*$_3$ Intermediate 4 is shown in blue. The spectrum of the bovine *aa*$_3$ P$_{M}$ is shown in green. The intermediate spectra are referenced versus the respective MVCO enzyme.
Figure 24. A comparison of the spectrum of the intermediate formed upon the decay of $A_R$ in the reaction between dioxygen and the fully reduced $Rs$ $aa_3$ with the spectrum of bovine $F$, generated during the reaction between dioxygen and the fully reduced bovine heart $aa_3$. The $Rs$ $aa_3$ intermediate spectrum is shown in blue. The spectrum of the bovine $aa_3$ $F$ is shown in green. The intermediate spectra are referenced versus the respective FRCO enzyme.
compound $\mathbf{AR}$ decays to $\mathbf{F}$ during dioxygen reduction in the fully reduced $Rs \ aa_3$, it is clear that the reaction of the mixed-valence $Rs \ aa_3$ with $O_2$ terminates at intermediate $PM$ following the decay of $AM$. Based upon the good agreement between the intermediate spectra of the mixed-valence $Rs \ aa_3$ and bovine enzymes, a mechanism for the reaction between dioxygen and the mixed-valence $Rs \ aa_3$ may be proposed (Figure 25) that is analogous to the reaction of the mixed-valence bovine $aa_3$ with $O_2$.

Our results show that the second-order rate constant for $O_2$ binding in the mixed-valence $Rs \ aa_3$ is approximately two times slower than in the fully reduced enzyme, which can be accounted for by the significant electron back-flow in the mixed-valence $Rs \ aa_3$ [39]. However, in the mixed-valence $Ec \ bo_3$, the rate of $O_2$ binding is similar (the rates differ by a factor of 1.5) to the fully reduced enzyme, which is consistent with the small extent of electron backflow previously observed in the mixed-valence $Ec \ bo_3$ [39]. Furthermore, splitting of the $O_2$ double bond is significantly slower in the mixed-valence $Ec \ bo_3$ (142 $\mu$s) than in the fully reduced enzyme (22 $\mu$s) [23]. Likewise, dioxygen splitting occurs on a longer time scale in the mixed-valence $Rs \ aa_3$ (470 $\mu$s) than in the fully reduced $Rs \ aa_3$ (53 $\mu$s) [22]. This is also the case for the mixed-valence bovine $aa_3$; dioxygen splitting occurs with a lifetime of $\sim 160 \mu$s in the mixed-valence bovine enzyme versus $\sim 35 \mu$s in the fully reduced bovine enzyme [40-42]. Our recent studies on dioxygen reduction in the fully reduced $bo_3$ have shown that $O_2$ binding to heme $a_3$, which occurs with a lifetime of $40 \mu$s, is followed by breakage of the $O_2$ double bond with an apparent lifetime
**Figure 25.** Proposed mechanism for the reaction of the mixed-valence $Rs\ aa_3$ with $O_2$. 

$$
\begin{align*}
&\text{R}_M \\
&\quad \text{TyrOH} \\
&\quad a_3^{2+} \quad Cu_B^+ \\
&\quad a^3+ \quad Cu_A^{2+} \\
&\quad \xrightarrow{31\ \mu s} \\
&\quad +O_2 \\
&\quad \text{TyrOH} \\
&\quad a_3^{2+} \quad Cu_B^+ \\
&\quad a^3+ \quad Cu_A^{2+} \\
&\quad \xrightarrow{470\ \mu s} \\
&\quad H^+ \\
&\quad \text{TyrO}^+ \\
&\quad (a_3=O)^{2+} \quad -HO-Cu_B^{2+} \\
&\quad a^3+ \quad Cu_A^{2+} \\
&\quad \text{Int 2} \\
&\quad \xleftarrow{2\ \mu s} \\
&\quad \text{A}_M \\
&\quad \text{P}_M
\end{align*}
$$
of 22 µs, which is ~6 times faster than observed for the reaction of the mixed-valence bo3 with O2 [23]. The different redox states of the high-spin heme in the mixed-valence and fully reduced enzymes may account for the different rates of dioxygen splitting. In the reduced bo3 and aa3 enzymes, two of the four electrons required for oxygen splitting come from the high-spin heme, one electron from CuB+, and one from the reduced low-spin heme. However, in the mixed-valence enzymes, the low-spin heme is oxidized and is unable to act as an electron donor during oxygen splitting; it has been suggested that the fourth electron originates from a cross-linked tyrosine residue [43, 44].

In the Ec bo3, the intermediate formed upon the decay of compound A is primarily F for both the fully reduced enzyme (P/F: 30/70) [23] and the mixed-valence enzyme (P/F: 40/60). This finding contrasts with the mixed-valence Rs aa3 enzyme, in which the reaction terminated with intermediate PM upon the decay of AM; however, during dioxygen reduction in the fully reduced Rs aa3, compound A decayed directly to F, with no detectable formation of PR [22]. Intermediate PM is also the terminal intermediate formed following the decay of AM in the reaction of the mixed-valence bovine aa3 with O2 [42]; however, in the fully reduced bovine aa3, the intermediate generated upon the decay of AR is a pH-dependent mixture of compound AR, PR and F [24]. It has been suggested that intermediates PR (or PM) and F differ in their protonation states [22]; presumably, a protonable functional group within the heme-copper oxidase donates a proton to the binuclear center during the conversion of PR (or PM) to F. The 40/60 mixture of PM and F observed in the reaction between
the mixed-valence Ec bo3 and dioxygen may arise from different enzyme populations. In the more abundant Ec bo3 population, this protonable group may be protonated and able to deliver H+ to the active site, generating F; however, in the less abundant enzyme population, this functional group may be unprotonated and unable to donate H+, resulting in PM.

**Conclusions**

Our findings suggest that breaking of the O2 double bond generates a 40/60 mixture of PM/F with a lifetime of 142 μs during the reaction of the mixed-valence Ec bo3 with dioxygen. In previous studies on the fully reduced Ec bo3, compound A decayed to a 30/70 mixture of P/F (with a lifetime of ~30 μs) prior to the formation of F [23]. Thus, in both the fully reduced and mixed-valence forms of Ec bo3, the primary intermediate generated following splitting of dioxygen is the oxyferryl F intermediate. However, in Rs aa3, A_R decays to F [22], whereas A_M decays to P. It has been suggested that P and F differ in their protonation states [22]. If a protonable functional group donates a proton during the conversion of PR to F in Rs aa3 and Ec bo3, then the detection of PM as the final intermediate during the reaction of the mixed-valence Rs aa3 with dioxygen suggests that this functional group may not be protonated in the mixed-valence Rs aa3 enzyme.
References


22. Szundi, I., Funatogawa, C., Cassano, J., McDonald, W., Ray, J., Hiser, C., Ferguson-Miller, S., Gennis, R. B. & Einarsdottir, O. (2012) Spectral identification of intermediates generated during the reaction of dioxygen with the wild-type and EQ(I-


29. Rumbley, J. N., Furlong Nickels, E. & Gennis, R. B. (1997) One-step purification of histidine-tagged cytochrome bo3 from *Escherichia coli* and demonstration that
associated quinone is not required for the structural integrity of the oxidase, *Biochim Biophys Acta.* **1340,** 131-42.


Appendix

Singular Value Decomposition, Global Exponential Fitting, and Intermediate Spectra

**Singular Value Decomposition (SVD)**

Multi-wavelength time-resolved optical absorption (TROA) spectroscopic studies generate large quantities of data which are often difficult and time consuming to analyze. The TROA difference spectra (post- minus pre-photolysis) acquired at various wavelengths ($\lambda$) and time delays ($t$) may be represented as the data matrix $\Delta A(\lambda, t)$, where $\lambda$ and $t$ determine the dimensions of the data matrix. SVD is utilized as a tool to reduce the size of the original $\Delta A$ data matrix into a truncated version which retains important spectral information [1, 2]. The SVD analysis decomposes the original $\Delta A$ data matrix into a product of three orthogonal matrices $U$, $S$, and $V$ [1, 3-5]:

$$\Delta A = USV'$$

The $U$ matrix represents the orthonormal basis spectra and $V'$ is the transpose of $V$, an orthonormal matrix which contains the time evolution of the $u$-spectra [1, 3]. The diagonal $S$ matrix is composed of the significance (singular) values, which show the contributions of the $u$ and $v$ vector pairs to the data matrix. Because every experimental data set contains a limited number of chemical intermediates, the data
matrix can be reconstructed with a limited number of \( u \) and \( v \) vector pairs with a significance value above the level of experimental noise. The SVD analysis significantly reduces the number of kinetic traces subjected to the global exponential fitting by only utilizing the significant \( u, s, \) and \( v \) vectors. SVD also enables the number of spectrally unique intermediates in a kinetic process to be estimated [6].

**Global Exponential Fitting and Intermediate Spectra**

For first-order or pseudo-first-order reactions, the time-dependence of the data matrix can be described by a sum of exponential functions:

\[
\Delta A(\lambda, t) = \sum_{i=0}^{N} b_i(\lambda) e^{-k_i t} + b_0(\lambda)
\]

where \( N \) is the number of exponentials; the \( k_i \) values are the apparent rate constants, usually represented in terms of lifetime, \( \tau (1/k_i) \); and \( b_i(\lambda) \), referred to as \( b \)-spectra, represent the spectral amplitudes associated with the respective rate constants. The \( b_0(\lambda) \) term is the difference spectrum extrapolated to infinite time [5, 7]. The \( b \)-spectra and apparent rates are determined for the SVD-filtered experimental data set by fitting the time-dependence of the \( V \) matrix to a sum of exponentials using Matlab (Mathworks).

By themselves, the apparent rates and \( b \)-spectra obtained from a global exponential fit do not represent the microscopic rates and spectral differences between individual intermediates. A reaction scheme must be proposed to connect the
apparent rates with the microscopic rates, and the $b$-spectra with the experimental intermediate spectra [7, 8].

The simplest reaction scheme is a unidirectional sequential mechanism in which the experimental apparent rates are assigned as the microscopic rates, in order from the shortest to the longest lifetime [7], such as in the mechanism below:

\[ \text{Int 1} \xrightarrow{k_1} \text{Int 2} \xrightarrow{k_2} \text{Int 3} \xrightarrow{k_3} \text{Int 4} \xrightarrow{k_4} \text{Int 5} \]

We employ an algebraic approach to describe a reaction mechanism. A set of linear first order differential equations describing the change in the concentrations of the intermediates with time is:

\[
\frac{dc}{dt} = K \ast C
\]

where the $\frac{dc}{dt}$ matrix represents the derivative of the concentrations of all intermediates; the $K$ matrix contains the rate constants of all the steps; and the $C$ matrix contains the concentration of each intermediate at a specific time.

The microscopic rate constants for a straightforward unidirectional sequential mechanism are arranged in a kinetic matrix, $K$, as follows:

\[
K = \begin{bmatrix}
-k_1 & 0 & 0 & 0 & 0 \\
 k_1 & -k_2 & 0 & 0 & 0 \\
 0 & k_2 & -k_3 & 0 & 0 \\
 0 & 0 & k_3 & -k_4 & 0 \\
 0 & 0 & 0 & k_4 & 0
\end{bmatrix}
\]

For more complex schemes, the $K$ matrix has more entries of reaction rate constants.
The solution to the set of differential equations above is a sum of exponentials:

\[ C(t) = \sum w_i e^{\alpha_i t} \]

where \( w_i \) are the eigenvectors and \( \alpha_i \) are the eigenvalues of the kinetic matrix, \( K \).

Thus, \( k_i = -\alpha_i \). The data matrix is as follows:

\[ \Delta A(\lambda, t) = E(\lambda)W e^{\alpha_i t} \]

where \( W \) is the matrix of the eigenvectors, \( w_i \), and \( E \) represents the intermediate spectra of a unidirectional sequential mechanism. Thus:

\[ b(\lambda) = E(\lambda)W \]

where \( b \) represents the \( b \)-spectra. The intermediate spectra, \( E \), of a unidirectional sequential reaction scheme are extracted from the experimental \( b \)-spectra using the eigenvector matrix, \( W \), of the kinetic matrix [7, 8]:

\[ E(\lambda) = b(\lambda)W^{-1} \]

The calculated experimental intermediate spectra are then compared to model spectra to test the validity of the proposed reaction mechanism.
References


