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Probing the structure-function relationship of heme c containing bacterial proteins: monoheme cytochromes c and diheme cytochrome c peroxidase

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Dissertation

PROBING THE STRUCTURE-FUNCTION RELATIONSHIP OF
HEME C CONTAINING BACTERIAL PROTEINS: MONOHEME
CYTOCHROMES C AND DIHEME CYTOCHROME C
PEROXIDASE

by

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This dissertation is dedicated to my family.

Thank you for all your love and support. I could not have accomplished any of this without you.
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PROBING THE STRUCTURE-FUNCTION RELATIONSHIP OF HEME C CONTAINING BACTERIAL PROTEINS: MONOHEME CYTOCHROMES C AND DIHEME CYTOCHROME C PEROXIDASE

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Boston University Graduate School of Arts and Sciences, 2013

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ABSTRACT

Heme containing proteins and their reactivity play a central role in biological systems; they have a vast range of functions including electron transfer, catalysis, and respiration. Cytochrome c and heme c containing proteins have been used widely as model systems to understand how structure and dynamics lead toward function. In this thesis, a variety of biophysical methods are used to investigate two heme c containing model systems to gain insight into how redox potential and reactivity are modulated through changes in the local environment.

Mitochondrial cytochrome c undergoes several pH dependent conformational rearrangements that involve different heme ligation and have associated changes in redox potential. Under basic conditions (pH greater than 8), the axial methionine (Met) residue is replaced by one of several nitrogen based ligands, usually a nearby lysine residue, and
is coined the “alkaline transition”. It is accompanied by a large downward shift in redox potential. The functional utility of this conformational change is not fully understood however it is strongly implicated in the signaling cascade for apoptosis. Bacterial monoheme cytochromes c exhibit similar phenomenological Met-loss behavior as a function of electrode material. In Chapter 2 we utilize *Hydrogenobacter thermophilus* cytochrome c552 as a model system for the assessment of redox thermodynamics and changes in redox potential associated with the Met-loss form. In Chapter 3 we extend our investigation to homologous cytochromes c.

Bacterial cytochrome c peroxidases catalyze the two-electron reduction of hydrogen peroxide to water utilizing cytochrome c as an endogenous electron donor. Chapter 4 describes the first recombinant construct of the diheme *Nitrosomonas europaea* cytochrome c peroxidase (Ne CCP): a defining family member of constitutively active cytochrome c peroxidases. A variety of biophysical techniques were used to confirm similarity between the recombinant Ne CCP and native enzyme. Chapter 5 extends our investigation to the role of constitutively conserved glutamine and glutamic acid residues within the active site, and two conserved tryptophan residues, the first situated between hemes and the second distal to the active site. In Chapter 6, stopped-flow spectroscopy is used to investigate the first intermediates of the Ne CCP catalytic mechanism.
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List of Abbreviations

AAS  atomic absorption spectrophotometer
A/D  analog-to-digital
AHT  anhydrotetracycline
AO   ascorbate oxidase
Au-SAM  a gold electrode modified with alkanethiol self-assembled monolayer
BCA  bicinchoninic acid
bCCCP  Bacterial cytochrome c peroxidase
CD   circular dichroism
CE   an electrochemical step following a chemical step with a comparable rate
CEC  an electrochemical step flanked by two chemical steps with comparable rates
Cm-  cytochrome c maturation genes
Cys  cysteine amino acid residue
CV   cyclic voltammetry
D/A  digital-to-analog
DMP  2,6-dimethylphenol
D. vulgaris  Desulfovibrio vulgaris
EC   an electrochemical step followed by a chemical step with a comparable rate
E. coli  Escherichia coli
ECD  electrochemical detection module
EDTA  ethylenediaminetetraacetic acid
EPR  electron paramagnetic resonance
FRA  frequency response analyzer module
gDNA  genomic DNA
HALS  highly axial (or anisotropic) low spin species
HCP  hybrid cluster protein
Hh   horse heart
HP-7 heme propionate-7
Ht   Hydrogenobacter thermophilus
HT   Hydrogenobacter thermophilus cytochromes c552
HT dm  Hydrogenobacter thermophilus cytochromes c552 M13/K22M
HX   hydrogen exchange
IPE  ideal reversible electrode
IRP  iron regulatory protein
i_{lim}  limiting current
LMCT  ligand metal charge transfer
MCD  Magnetic circular dichroism
Met-loss  cytochrome c species with the axial met dissociated
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)-propanesulfonic acid</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Ne</td>
<td><em>Nitrosomonas europaea</em></td>
</tr>
<tr>
<td>NE</td>
<td><em>Nitrosomonas europaea</em> cytochromes c552</td>
</tr>
<tr>
<td>O₂</td>
<td>Triplet state of molecular oxygen</td>
</tr>
<tr>
<td>OM bs</td>
<td>Mitochondrial ferric cytochrome b5</td>
</tr>
<tr>
<td>Pa</td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td>PA</td>
<td><em>Pseudomonas aeruginosa</em> cytochrome c551</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide slab gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data bank</td>
</tr>
<tr>
<td>PFV</td>
<td>Protein film voltammetry</td>
</tr>
<tr>
<td>PGE</td>
<td>Pyrolytic graphite edge-plane</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl methyl sulfonyl fluoride</td>
</tr>
<tr>
<td>P. furiosus</td>
<td><em>Pyrococcus furiosus</em></td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>RDE</td>
<td>Rotating disk electrode</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembled monolayer</td>
</tr>
<tr>
<td>S.C.E</td>
<td>Standard calomel electrode</td>
</tr>
<tr>
<td>S.H.E</td>
<td>Standard hydrogen electrode</td>
</tr>
<tr>
<td>yCCP</td>
<td>Yeast cytochrome c peroxidase</td>
</tr>
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</table>
Chapter 1 Introduction

1.1 Reactive oxygen species

Aerobic organisms, which derive their energy from the reduction of molecular oxygen (O$_2$), are unavoidably confronted with reactive oxygen species (ROS) and oxidative stress (1-3). Oxidative stress is the result an excess of ROS above and beyond the cellular capacity to provide an adequate response which results in oxidative damage. These ROS are formed through the incomplete stepwise reduction of molecular oxygen to water (Figure 1.1).

![Redox states of oxygen with standard potentials](image)

Figure 1.1 Redox states of oxygen with standard potentials. Standard reduction potentials are reported (3-5). Reactive oxygen species come in two general forms; free radical species such as superoxide (•O$_2^-$) and hydroxyl radical (•OH), and non-radical species including hydrogen peroxide (H$_2$O$_2$), hypochlorite (OCl$^-$), and peroxynitrite (ONO$_2^-$)(4-7). Deleterious effects include DNA damage, lipid oxidation, and destruction of enzyme cofactors (2, 3, 8).
Despite the harm ROS can cause, they are used in cell signaling and immune responses to combat invading organisms (6, 7). Cells have several ways of minimizing ROS toxicity through chemical antioxidants and enzymes. Aerobic respiration is approximately 18 fold more energetically profitable per glucose molecule than anaerobic respiration making ROS detoxification worth the energetic cost.

1.2 Generation of reactive oxygen species.

![Molecular orbital scheme for various states of molecular oxygen (Adapted from (9)).](image)

The triplet ground state of molecular oxygen ($^3\text{O}_2$) is thermodynamically favored over both singlet states ($^1\text{O}_2$). The spin aligned electrons are a general source of stability due to the necessity of a spin flip, and the reason combustibles do not spontaneously form the lowest energy products, CO$_2$ and water (9). This inherent stability breaks down in the presence of radicals, organic compounds, and transition metals; the unpaired electrons of $^3\text{O}_2$ can react with unpaired electrons (Figure 1.2).

During the late 1870’s Fenton described a color change associated with the mixing of Fe$^{2+}$ salts with tartaric acid and hydrogen peroxide (11, 12). Haber and coworkers in the 1930’s proposed the formation of hydroxyl radical from the peroxide/Fe$^{2+}$ interaction (11, 13). The disproportionation of hydrogen peroxide through
interaction with iron and the production of hydroxyl radicals is known as the Fenton Reaction (Figure 1.3).

\[
\begin{align*}
\text{Fe}^{2+} + \text{H}_2\text{O}_2 + \text{H}^+ & \rightarrow \text{Fe}^{3+} + \text{H}_2\text{O} + \text{HO}\cdot \\
\text{Fe}^{3+} + 1/2\text{H}_2\text{O}_2 + \text{H}^+ & \rightarrow \text{Fe}^{2+} + 1/2\text{O}_2 + \text{H}^+ \\
1/2\text{H}_2\text{O}_2 & \rightarrow \text{H}_2\text{O} + 1/2\text{O}_2 + \text{HO}\cdot
\end{align*}
\]

Figure 1.3 Fenton Chemistry: generation of hydroxyl radicals through the reaction of hydrogen peroxide.

Intracellular regulation of metal ions is very tightly controlled (14), however Fenton chemistry and ROS generation can occur at accessible protein transition metal centers. Hydroxyl radical formation can go on to do a variety of chemistry. In general, radical chemistry is responsible for many energetically difficult chemical transformations; among these transformations are oxygenation and hydrogen-atom abstraction (15). The ability to do these transformations is a consequence of high redox potentials and large driving force to accept electrons (16, 17).

The primary source of ROS generation is the aerobic respiratory machinery from the incomplete reduction of oxygen (4). For example, the primary production of superoxide and hydrogen peroxide may be mediated through the interaction of oxygen with the flavin domains of respiratory dehydrogenases (18, 19). Flavin represents a potential source of two electrons; the initial interaction with oxygen produces superoxide and the second hydrogen peroxide molecule (Figure 1.4). In the cases of succinate and
fumarate dehydrogenase, the oxidation of the flavin semiquinone is unfavorable, producing only superoxide (5, 7).

![Reduced Flavin and Oxygen](image)

![Oxidized Flavin and Hydrogen Peroxide](image)

**Figure 1.4** Hydrogen peroxide product production through the reduction-oxidation reaction of flavin and oxygen.

### 1.3 Detoxification of reactive oxygen species

Given the mechanisms for damage, the detoxification of ROS is of paramount importance for bacterial survival. There are three major classes of regulatory mechanisms: superoxide dismutase (SOD), catalases, and peroxidases (20, 21). SODs produce molecular oxygen and hydrogen peroxide from two equivalents of superoxide. Depending on the source and cellular localization, SODs contain a variety of redox active transition metals: copper-zinc, iron, manganese, and nickel. Here we will examine two heme based systems for the regulation of peroxide: catalases and peroxidases.
1.3.1 Catalases

Catalases remove hydrogen peroxide from cells through disproportionation to water and molecular oxygen. This class of enzymes may be divided into three groups: mono-functional, bi-functional catalase-peroxidase, and manganese-containing catalases (22). Both mono- and bi-functional catalases are heme-containing enzymes which share a two-step mechanism (Figure 1.5).

![Figure 1.5 Catalase mechanism for the disproportionation of hydrogen peroxide.](image)

Bi-functional catalases, like hydroperoxidase I (KatG) from *E. coli* have an additional low efficiency peroxidase activity (22, 23). The resting state of both heme catalases is a ferric five-coordinate heme. Upon binding of hydrogen peroxide a two-electron oxidation yields the first equivalent of water, an oxyferryl species, and heme cation radical, compound I. Interaction with a second equivalent of peroxide yields a second molecule of water and molecular oxygen (22).

Di-manganese enzymes disproportionate two molecules of hydrogen peroxide in two steps (Figure 1.6).

![Figure 1.6 Disproportionation of hydrogen peroxide by manganese-containing peroxidase.](image)
The first mechanistic step is similar to heme catalase where the metal is oxidized, but a highly reactive intermediate is not made. Instead of reducing the iron-oxo species, the second step is a reduction of the metal active site. Additionally, both waters are generated in the first step through the donation of two enzyme-derived protons (22).

1.3.2 Peroxidases

Peroxidases differ from catalases in their cellular localization and mechanism. Catalases are typically cytosolic while peroxides are found in the intermembrane spaces; periplasm and intermitochondrial spaces. The peroxidase reaction consists of two one electron reactions that may be coupled to a proton transfer. Additionally, peroxidases only use peroxide as a terminal electron acceptor, where catalases can utilize peroxide as both acceptor and donor. Among the peroxidase superfamily, there are two subgroups of heme peroxidases. Both eukaryotic and prokaryotic organisms contain monoheme peroxidases, while diheme cytochrome c peroxidases (CCPs) are found throughout Gram negative bacteria (24).

1.3.2.1 Monoheme peroxidases

Monoheme peroxidases typically contain a $b$-type heme cofactor, which is not covalently attached to the protein backbone (25-27). Figure 1.7 outlines the general mechanism for the reduction of hydrogen peroxide to water (24). The resting state of monoheme peroxidases are a five coordinate ferric heme. Hydrogen peroxide binds to the open site forming an iron-hydroperoxo species, known as compound 0. Rapid heterolytic cleavage of the O-O bond releases a single water molecule. Simultaneously the heme
Figure 1.7 General monoheme peroxidase catalytic mechanism. R is a stable porphyrin radical in most monoheme enzymes, and is Trp-191 in yeast cytochrome c peroxidase. The active site is oxidized by two electrons forming a high valent iron-oxo and a porphyrin radical species; compound I (28). The generation of compound I is facilitated by several conserved residues. Distal to the heme are a histidine (His) and arginine (Arg) residues. The distal His acts as a proton acceptor from hydrogen peroxide, while the Arg stabilizes the partial negative charge during the O-O bond cleavage (28, 29). Generation of compound I in the monoheme cytochrome c peroxidase from Saccharomyces cerevisiae (yCCP) is an exception to the canonical mechanism. The cation radical is translocated to a distal tryptophan (Trp) residue. The Trp residue is unique to yCCP (30). In canonical monoheme peroxidases, like horseradish peroxidase, the cation radical is porphyrin based, and this position is occupied by a phenylalanine residue (30). Additional reducing equivalents are provided by monoheme cytochrome c. The first equivalent quenches the
organic radical (compound II). The second is a proton coupled step that results in product release, in this case water.

Plant peroxidases and cytochrome P450 enzymes are also part of this family. These enzymes utilize the strongly oxidizing nature of the iron(IV)-oxo (compound I) to carry out oxidation of various substrates (31). It is important to note that P450’s activate molecular oxygen and function as monooxygenases. However their reactive intermediate, an iron(IV)-oxo and porphyrin cation, is similar to that of monoheme peroxidases. Their active sites are deeply buried in the protein matrix, and their proton delivery system is more complex; utilizing conserved threonine, asparagine, lysine, and arginine residues (31).

1.3.2.2 Diheme peroxidases

Bacterial diheme peroxidases (bCCPs) were initially reported in the early 1970’s by Ellfolk and Soininen (32, 33). In contrast to the eukaryotic enzymes of the superfamily, the bCCP from Pseudomonas aeruginosa (Pa) contained two covalently attached c-type hemes in functionally unique domains (34, 35). The non-equivalence of these hemes became readily apparent though further spectroscopic studies which helped develop a catalytic model (34-36). Electronic absorption spectroscopy (UV-visible spectroscopy, magnetic circular dichroism (MCD), etc.) and electron paramagnetic resonance (EPR) were used to identify crucial characteristics of each center such as electrochemical potential, spin state, and ligand environment. Determination of the reduction potential revealed a nearly 650 mV separation between hemes (37). The high
potential (H) heme has a midpoint of +320 mV vs. SHE while the low potential (L) heme has a reduction potential of -320 mV vs. SHE (34, 35). This clear separation suggests some insight into the functional roles of each site. The H-heme can serve as an acceptor from electron donors, most often monoheme cytochrome c or blue copper proteins such as azurin. The H-heme can then act as a conduit for electrons shuttled to the L-heme. The L-heme serves as the catalytic site where hydrogen peroxide binds and is reduced. Notably, for the Pa CCP, the rate of catalysis was observed to be orders of magnitude higher when starting in the mixed valent, or semi-reduced form (Fe\text{LP}^{3+}Fe\text{HP}^{2+}), than the as isolated diferric form (Fe\text{LP}^{2+}Fe\text{HP}^{3+}) (38-40).

The functional model was further refined by examining redox-dependent changes correlated with catalysis. In the diferric form, EPR spectra indicate that the H-heme is in spin equilibrium between high and low spin states, while the L-heme is low spin. Upon formation of the Fe\text{LP}^{3+}Fe\text{HP}^{2+} (semi-reduced) state, the H-heme becomes low spin and the L-heme becomes five coordinate and high spin. A peroxide binding site becomes readily accessible by removal of a ligand and the enzyme is reductively active. Taking into account two heme moieties and an activation process upon semi-reduction, a catalytic scheme was proposed (Figure 1.8) as described below.

\textit{Pa CCP} is activated from the diferric to semi-reduced form. During activation, a loss of an axial histidine occurs at the L-heme allowing substrate access. Hydrogen peroxide may then bind to the open coordination site, generating a compound II-like intermediate. Due to the storage of a reducing equivalent in the H-heme generation of an
organic radical (porphyrin or tryptophan) is not necessary. Upon binding, peroxide may go through a hydroperoxo intermediate to ultimately generate the iron(IV)-oxo species. A proton coupled electron transfer forms a hydroxo intermediate which may be further protonated to release the second equivalent of water. A final reducing equivalent brings the enzyme back to the resting state.

![Catalytic mechanism of Pseudomonas like diheme peroxidases.](image)

Structural studies of *Pa* CCP and other bCCP family members support the spectroscopic and catalytic findings outlined above (Figure 1.8), and *Pa* structures can be used to highlight features found amongst all of the bCCP family. *Pa* CCP exists as a homodimer with each protomer containing the two c-heme cofactors. A calcium binding site was found between the two sub-domains and shown to be essential for catalysis. The diferric models of the *Pa* bCCP showed the H-Heme to be His-Met ligated and the L-Heme to be bis-His coordinated (Figure 1.9A). Semi-reduction causes a series of loop rearrangements ultimately resulting in removal of the axial histidine (Figure 1.9B).
Figure 1.9 Crystalographic models of *Pseudomonas aeruginosa* cytochrome c peroxidase monomers. (A) Diferric form PDB: 1EB7 (B) Semi-reduced form PDB:2VHD. Yellow spheres represent interdomain Ca$^{2+}$ ions. Redox linked structural changes are highlighted. Loop I is colored in red and the axial His ligand is shown.
Comparison of amino-acid sequences by multiple sequence alignment show an overall high sequence identity across the diheme CCP family (Table 1.1). Among the available crystallographic models the backbone alpha carbon RMSD values are approximately 0.85. Loops I, II, and III identified in the Pa CCP structures represent highly conserved regions among the bacterial diheme peroxidases. Other important features are the two c-type heme binding motifs and calcium-binding site. Recent studies have underscored the importance of the calcium site for dimerization in addition to proper activation. The location of the Ca$^{2+}$ and necessity for reactivity suggests a key role for proper electronic communication between hemes. Important residues may be more easily identified by conservation among the many family members in multiple sequence alignments (Figure 1.10).
Figure 1.10 Sequence alignment of various bacterial cytochrome c peroxidases.
1.3.2.3 Constitutively Active Diheme peroxidases

In contrast to the canonical bCCPs, like Pa CCP described above, the enzyme from *Nitrosomonas europaea* was the first bacterial CCP to be fully active in the diferric form. The initial purification and characterization showed that upon reduction of the H-heme, peroxidase activity fell by two orders of magnitude using horse heart cytochrome c as an electron donor (41). The *N. europaea* CCP (Ne CCP) EPR and UV-Visible spectroscopy revealed that in the diferric form, the H-heme was six-coordinate and low spin, while the L-heme was five-coordinate and high spin. Crystallographic analysis of the Ne CCP structure (Figure 1.11) clearly accounts for the spectroscopic findings and shows differences in structure, particularly the solvent exposed L-heme (42).

![Figure 1.11 Model of *N. europaea* cytochrome c peroxidase indicating that the low potential heme is solvent exposed in the diferric state. Loop 1 highlighted in red, and hemes are shown in orange. Models shown (A) with surface rendering and (B) without. PDB 1IQC.](image)

The bCCP from *Methylococcus capsulatus* Bath is also fully active in the diferric form (43). This is not surprising based on the similarities of metalloproteins studied from
ammonia-oxidizing and methane-oxidizing bacteria. Protein film voltammetry (PFV) was used to characterize the catalytic electrochemistry of the native Ne CCP. Since the enzyme is most active in the fully oxidized form, the high potential heme is likely innocent in the initial catalytic steps (44, 45). This further suggests the possibility of the existence of a compound I-like species during the course of catalysis. In a combination of the initial spectroscopic (41) and PFV analysis, the catalytic scheme in Figure 1.12 was proposed (45).

![Catalytic mechanism of *Nitrosomonas europaea* like diheme peroxidases.](image)

Further examination of the Ne CCP active site reveals residues that could aid in proton coupled electron transfer steps and the formation of a compound I-like species. Gln92 and Glu102 are positioned such that their side chains point directly toward the center of the L-heme (42). These residues could potentially serve similar roles as the distal His and Arg discussed in the formation of compound I for monoheme cytochrome c peroxidases.
Interestingly, corresponding residues in Pa CCP shift dramatically depending on the oxidation state to an edge to edge distance as little as 5 Å of the heme iron when semi-reduced.

1.4 Aims of this thesis

The work presented in this thesis is focused on the structure-function relationships of proteins containing c-type hemes. Two model systems have been explored herein, bacterial monoheme cytochromes c that are prototypical systems for studying electron transfer, and the monoheme cyt c that are analogous to mitochondrial cyt c have been studied to understand the relations between redox chemistry and potential, and structural dynamics. The second model system is the bacterial diheme peroxidase from Nitrosomonas europaea described in section 1.3.2.3 above. Below I describe the remaining chapters of this thesis.

Chapter 2. Methionine ligand lability in Hydrogenobacter thermophilus cytochrome c552

Electrochemical investigations of monoheme cytochromes c by protein film voltammetry (PFV) revealed an additional low potential equilibrium, previously unobserved as a function of electrode material. This chapter focuses on electrochemical analysis to determine the nature of the low potential, methionine-loss species observed in Hydrogenobacter thermophilus (Ht) cytochrome c552. The native methionine is shown to be susceptible to replacement by the small molecule imidazole. Mutations of the axial methionine can be made to mimic the low potential methionine-loss feature. Redox thermodynamics of the native and mutations are consistent with those of other
cytochromes. Finally, the required driving force for the rearrangement may be enhanced by the binding of cytochrome c to a graphite surface.

Chapter 3. Methionine ligand lability of orthologous cytochromes c

Electrochemically detected thermodynamic analyses of monoheme cytochromes c from *Shewanella oneidensis*, *Nitrosomonas europaea*, and *Pseudomonas aeruginosa* are compared in this chapter, to extend the findings of Chapter Two. The temperature dependent measurements show systematic changes in the normal and low potential features similar to those observed for *Ht* cyt c. The populations of these features inform the relative driving force for the conformational change.

Chapter 4. Production and characterization of recombinant *Nitrosomonas europaea* cytochrome c peroxidase.

Native *Nitrosomonas europaea* cytochrome c peroxidase has been studied previously, but until now there has been no recombinant system for expression. Here, a new system for *N. europaea* cytochrome c peroxidase expression that is optimized for *E. coli* codon usage is described. This chapter will focus on the full characterization of the oligomeric state. Activity will be confirmed by solution assays utilizing both horse heart cytochrome c and the *Nitrosomonas europaea* cytochrome c552. Heme content is confirmed by pyridine hemochrome assay and atomic absorption spectroscopy. Experiments are conducted to determine the necessity for the interdomain Ca$^{2+}$ ion that
mediates dimerization as observed in other BCCPs and conformational changes required for activation of *Pseudomonas* like enzymes.

Chapter 5 A kinetic and electrochemical investigation into the roles of conserved tryptophan, glutamine, and glutamic acid residues in *Nitrosomonas europaea* cytochrome c peroxidase.

This chapter focuses on electrochemical and solution kinetic analysis to elucidate the role of conserved tryptophan residues. Trp61 is located distal to the low potential catalytic active site and Trp82 between the high and low potential hemes. Both Trp residues are conserved across all bCCPs and are found in segments of protein sequence that are highly conserved across all diheme cytochrome *c* peroxidases, despite the variety in structure and mechanism noted above. Trp61 represents the most direct route for electron transfer between the HP and LP hemes. Trp82 may function as the repository for one reducing equivalent, and may also be involved in projecting loop I to the second protein in bCCP dimers. Systematic mutations to tyrosine, phenylalanine, and alanine are conducted in order to yield small perturbations in structure, while enabling the assessment of the role of Trp in catalysis. This study sets the stage for further kinetic characterization of mutations in the LP heme pocket to residues, Q92N, E102D, and the double mutation Q92N E102D are of particular interest, as these mutations target the ability to deliver protons to the active site, potentially prolonging the lifetimes of
intermediate species, and aiding their future identification. Activity assays utilize both horse heart cytochrome c and the *Nitrosomonas europaea* cytochrome c$_{552}$.

**Chapter 6. Stopped-flow kinetics investigation of peroxidase intermediates in *Nitrosomonas europaea* cytochrome c peroxidase.**

In this chapter, an investigation is presented of the kinetic intermediates of the reaction of *N. europaea* CCP with peroxide. Historically, knowledge of the catalytic mechanism comes from stopped-flow spectroscopy, as in the case of the *Pseudomonas aeruginosa* CCP, which requires reductive activation to achieve a Fe$_{\text{HP}}^{2+}$Fe$_{\text{LP}}^{3+}$ oxidation state that is catalytically competent. This work suggests the formation of iron(IV)-oxo and iron(III)-hydroxyl intermediates. At present there has been no evidence for a hydroperoxy intermediate, like those in eukaryotic CCPs, preceding the other intermediates. *N. europaea* CCP is a member the group of bCCPs that do not require reductive activation, where the initial active oxidation state is Fe$_{\text{HP}}^{2+}$Fe$_{\text{LP}}^{3+}$. Stopped-flow optical measurements in both single wavelength, and diode array mode are presented in this chapter to determine the peroxide dependent features and rates of elementary steps, in order to develop a kinetic model for the reactivity of the enzyme with substrate. Overall we have examined the first kinetic intermediates by stopped-flow spectroscopy of *N. europaea* CCP in the diferric form with hydrogen peroxide. These studies are designed to provide a more comprehensive knowledge how *N. europaea* family of peroxidases
function, alongside our broader goal of understanding multi-electron chemistry in biological systems.
Chapter 2  Methionine ligand lability in bacterial monoheme cytochromes c from *Hydrogenobacter thermophilus*

This work has been previously published in the *Journal of Physical Chemistry B*, 2011, 115 (40), 11718-11726.

2.1 Introduction

Iron-protoporphyrin IX (heme) containing proteins occupy vast and diverse areas of biological function, where the heme cofactor frequently acts as an electron transfer element functioning in respiration, catalysis, and signaling. The ubiquitous nature, robust structure and spectroscopic signatures of heme proteins are the traits that have driven these proteins to be amongst the most studied biomolecules. Folding, dynamics, and function of the various proteins have prompted analyses through the sequence-structure-function paradigm (46-49) where the functional characterization of individual proteins is often described by the heme Fe(III/II) redox potential ($E_m$). Several factors have been strongly implicated in controlling the potential, particularly axial ligation and the second sphere interactions such as hydrogen-bonding and solvation (50-54): in such models, $E_m$ is coarsely determined by the axial coordination and further tuned by secondary interactions upon the axial ligands and heme pocket environment (50, 55-59).

Cytochromes c (cyts c) are additionally complex due to the covalent attachment of the porphyrin ring to the protein backbone through two thioether linkages in a -Cys-X-X-Cys-His- binding motif, where the histidine (His) serves as the proximal heme ligand (53, 55, 60), while a potential sixth ligand (such as a distal methionine (Met)) is donated from elsewhere in the protein scaffold. Here we consider the *Hydrogenobacter thermophilus*
(Ht) cytochrome c552, which displays a canonical cyt c fold (Figure 2.1) (53, 60, 61); Ht cyt c552 and its variants have been characterized extensively to further develop an understanding of how the heme attachment motif and heme pocket residues affect midpoint potential and dynamic properties (55-59, 62-66). In this report, we utilize the Ht cytochrome as a model system for the assessment of redox thermodynamics and changes in midpoint potential associated with the loss of the Met ligand.

![Figure 2.1 Cartoon representation of HT cyt c552 constructed from Protein Data Bank ID:1YNR (61) utilizing PyMOL. The heme c and axial ligands, His16 and Met61, are displayed as sticks. Axial ligands His16 (bottom) and Met61 (top) are highlighted in red. The Met-donating loop, loop3 is highlighted in blue.](image)

Recent electrochemical investigation of bacterial cytochromes c by protein film voltammetry (PFV) revealed not only the Fe(III/II) potential of the typical His/Met-bound form of the active site, but a second electrochemical equilibrium, which could be detected as a function of electrode material (57). While Ht cyt c552 as well as the Pseudomonas aeruginosa (Pa) cytochrome c551 exhibit a single electrochemical feature when adsorbed onto modified-gold electrodes (Au-SAM electrodes) (58) a second
reversible couple at approximately -100 mV (vs hydrogen at pH 7) was observed upon pyrolytic graphite edge (PGE) electrodes (Figure 2.2), a phenomenon that has been now confirmed for a small number of bacterial c-type cytochromes (67). The second redox couple was found to be due to a species that was best described as a locally-unfolded, or “Met-loss” state, via comparisons with chemically unfolded protein (57) and correlation of the yield of the Met-loss form with thermal loss of intensity of a 695-nm absorption feature in the optical spectra of the cytochromes studied (14). Interrogation of the Met-Fe(III/II) interaction (68-72) and the structural implication of Met displacement (73-77) have underpinned the interplay between global and local environment in controlling the redox potential and thermodynamics. Superficially, the loss of Met as a ligand in the bacterial cyt c may be analogous to the changes observed in mitochondrial cyt c at high pH values (74, 78-80), i.e., the so-called “alkaline transition” which is marked by a
replacement of the distal Met ligand with a nitrogenous base (typically one of three lysine residues contained within the methionine donating loop of the protein scaffold (62, 81-84)) but an overall retention of global structure (73, 74). In contrast, cytochrome \( \text{c}_2 \) proteins from purple bacteria contain axial Met as a ligand, but with additional “hinge-like” regions on either side of the methionine-bearing loop, allowing for the protein structure to be largely unaffected by the local conformational changes resulting from small molecule binding (75, 85-87). Similarly, alkaline-like conformational changes have been demonstrated in the presence of membrane phospholipids, where interaction of cardiolipin with the solvent exposed heme propionates has been implicated in stimulating conformational changes and the lowering of \( E_m \) by several hundred millivolts (76, 77, 88-90).

However, the analogy to the alkaline transition fails in the case of our prior reporting of an electrochemically detected “Met-loss” state, as the bacterial homologs of mitochondrial cytochrome \( c \) all lack the analogous Lys residues that might serve as ligands. Critically, by all estimates, the resulting shift in heme iron coordination synonymous with the alkaline transition yields a new state that possesses a reduction potential even lower in potential(50, 57, 78-80, 90-92) than that which we and others have reported for bacterial cytochromes \( c \) upon PGE electrodes (57, 67). While previous efforts to characterize loop- and ligand-reorganization of Met-ligated cys \( c \) have correlated global stability with maintenance of Met-Fe coordination (using thermal denaturation experiments coupled to NMR and optical techniques),(93-95) (96) here we
further our prior studies of the link between protein dynamics and loss of the Met-ligand in bacterial cyts c through the technique of direct electrochemistry.

Presently we have conducted a systematic, variable-temperature, and ligand-binding electrochemical study to interrogate the low potential Met-loss feature associated with the bacterial Ht cyt c on PGE electrodes. Mutation of the axial methionine to alanine or histidine has allowed us to impose specific coordination of the heme-porphyrin moiety. We have utilized protein film voltammetry (PFV) to directly report upon relative populations of Met-ligated and Met-loss forms. Midpoint potentials agree well with models and similar mutations from other cyts c (50, 71, 80, 97-99). Temperature-dependent voltammetry has allowed the comparison of enthalpy and entropy for both the normal and alkaline forms of various cytochromes. Redox thermodynamics of our system correlate well with previously reported values which serve as further validation for our findings (80, 91, 92, 94, 98, 100). Additionally, as a result of having two distinct populations on a single surface we were able to determine the Gibbs free energy for the conformational change. Small molecule binding has been used to inform on the relative accessibility of the heme, which further elucidates the relationship between structure and the redox chemistry in the context of the lability of the Met61 ligand.

2.2 Materials and Methods

*Protein Production and Purification.* The mutation M61H was prepared by the polymerase chain overlap extension method (101). The mutation M61H was prepared by
QuikChange XL site directed mutagenesis Kit (Stratagene). pSCH552 was used as the DNA template and 5' GGG GTT CTG TTC CCC ACC CTC CTC AAA ATG TAA CCG ATG CG 3' and 5' CGC ATC GGT TAC ATT TTG AGG AGG GTG GGG AAC AGA ACC CC 3', were used as forward and reverse mutagenic primers, respectively. Overexpression was achieved by *E. coli* BL21(DE3) culture containing the appropriate pSCH552 plasmid and pEC86, containing the cytochrome *c* maturation genes *ccmABCDEFGH* (102). Expression of protein samples and purification procedures of Ht cyt *c* and mutants, M61A and M61H, were as previously described (58, 103).

**Electrochemical Methods.** All PFV measurements were carried out on a PGSTAT30 AutoLab (Ecochemie) electrochemical analyzer equipped with FRA and ECD modules. A water-jacketed glass cell was used in a three electrode configuration. Cell temperature was maintained by refrigerating circulator. A resin-body calomel electrode (Accumet) and platinum wire were used as the reference and counter electrodes respectively. The calomel reference was maintained at a constant temperature (293 ± 0.5 K). Potentials are reported vs. standard hydrogen electrode (SHE) and corrected by +242 mV. Nonfarradaic (charging) components of the resulting voltammograms were removed through polynomial baseline subtraction. Regular electrochemical noise was suppressed through Fast Fourier Transformation. Data analysis was done utilizing the open source program SOAS (104).

Pyrolytic edge-plane graphite (PGE), or polycrystalline gold wire (2.0 mm diameter) embedded in resin served as the working electrode. The gold electrodes were
polished with successively finer grits of alumina (1.0, 0.3, 0.05 μM, Buehler). Final
electrochemical cleaning was carried out in 0.1 M H₂SO₄ by cycling 0.2 – 1.35 V (vs
SCE). Cleaned surfaces were modified in an 0.5-2 mM ethanolic solution of 6-
mercaptohexanol for 12 hours. Excess alkane thiol was removed by rinsing with ethanol
followed by water and then considered ready for use. PGE electrodes were polished with
aqueous 1μ alumina slurry, followed by sonication. Protein films were generated on both
surfaces by directly applying 2 μL cytochrome c. Excess protein was washed away with a
small amount of cold buffer.

Thermodynamics of electron transfer was interrogated by variable-temperature
experiments over a limited range of temperatures (0 – 65°C). Reaction entropy (ΔS°rc)
for
the Fe (III/II) couple may be determined by the relation:

\[ \Delta S_{rc}^{°} = S_{red}^{°} - S_{ox}^{°} = nF \left( \frac{dE^{°}}{dT} \right) \]  

With the assumption that ΔS°rc is constant over the temperature range investigated, the
slope of the Em versus T should be linear and equal to ΔS°rc. Enthalpic change may be
obtained based on the previous assumption. From the Gibbs-Helmholtz equation, ΔH°rc
may be extracted from the negative slope of an E_m/T versus 1/T plot.

The populations of Met-loss and normal forms of Ht cyt c are represented in one
of two ways: either as the peak area (total charge passed) for each of the two redox
couples, or as a quantity proportional to the peak height of each redox couple in a CV.
The peak height formalism for an anodic (oxidation, \( i_{pa} \)) or cathodic (reduction, \( i_{pc} \)) feature is shown in equation 2(105).

\[
i_p = \frac{n^2 F^2 A \Gamma \nu}{4RT}
\]  

(2)

Here, \( n \) is the number of electrons, \( F \) is the Faraday constant, \( R \) is the ideal gas constant, \( T \) is the temperature, \( A \) the physical surface area, \( \Gamma \) total electroactive coverage, and scan rate \( \nu \). For a process with \( n=1 \), the population is directly proportional to the observed current. Temperature dependent behavior may be assessed using the equilibrium form of the Gibbs free energy equation and linear form of the van’t Hoff equation (equation 3):

\[
\ln K_{eq} = -\frac{\Delta H^o}{RT} + \frac{\Delta S^o}{T}
\]  

(3)

The equilibrium constant \( K_{eq} \) may be determined at each temperature by the ratio of Met-loss to normal form, and a plot of \( \ln(K_{eq}) \) versus \( T^{-1} \) allows the extraction of the thermodynamic parameters. Here we assessed \( K_{eq} \) in terms of both the peak area and peak height of each redox couple. \( \Delta G^o \) values were calculated at 298K.

Imidazole binding studies were carried out in 50 mM phosphate/citrate buffer, pH 6.0, containing varying amounts of imidazole (0 – 1 M). Electrodes with protein films were maintained in chilled buffer between measurements. All buffers were purged of oxygen with argon prior to measurement. Determination of the association constants for imidazole \( (K_a) \) were conducted in the manner of Sutin and Yandell (106).
2.3 Results

We have continued our previous work on the Ht cyt c protein as a model system for interrogating structure-function relationships that impact redox properties, such as the reduction potential. As in our prior efforts (56-58), we have found that the Ht wild type (WT) protein, reproducibly gives excellent electrochemical responses at PGE electrodes; however, as observed previously, the Ht WT protein exhibits two one-electron features: the typical redox couple observed at +210 mV, and what we have called the “Met-loss” form at -110 mV (Figure 2.2). To probe the hypothesis that the minor component observed is indeed a “Met-loss” state, and to interrogate whether other potential axial ligands might be present, we have prepared the M61A mutant, presuming that removal of the axial methionine by mutation to an alanine will provide an open coordination site that may be occupied by another exogenous ligand or, in the absence of exogenous small molecules, water/hydroxide may occupy the position of a sixth axial ligand. Importantly, the methionine donating loop of Ht does not contain another nitrogenous amino acid (*i.e.*, lysine) that could be poised to replace the thioether bond of methionine, as has been observed in eukaryotic cyts c(61). At 0 °C the presence of the low-potential, Met-loss form is sufficiently suppressed such that nearly the entire electrochemical response is due to the normal form of the protein, observed at +210 mV (Figure 2.3A). Upon removal of M61 in the M61A mutant, we observe a single feature: a one-electron redox couple at the dramatically lower potential of -163 mV (Figure 2.3B). Similarly, electrochemical characterization of the M61H mutant similarly resulted in simple voltammetric responses
that display a single redox couple (Figure 2.3C) with midpoint potential of -103 mV (and no indication of any additional features), as the M61H mutant is most likely a stable six-coordinate bis-His ligated heme. In all cases, there is a deviation from ideal electrochemical behavior of an adsorbed species: while all features appear symmetric and largely reversible, a significant (10-20 mV) peak separation is observed at 0 °C. In all cases, higher temperatures restored reversibility as peak separation tended toward zero, suggesting that at 0 °C a dispersed population of cytochrome c conformations were “frozen” on the PGE surface at low temperatures, giving a distribution of electron transfer rates and potentials that could be averaged out at higher temperatures. Notably, for all of these experiments the ratio of peak current ($i_{pa}/i_{pc}$) observed for each species is unity, over a wide range of scan-rates (from 20 mV/s to 20 V/s). Additionally we note that as a control, the electrochemistry of M61A was examined using mercapto-hexanol modified gold electrodes (Figure 2.4), as reported previously (56, 58), resulting in a single, reversible low-potential feature.
Figure 2.3 PFV response for (A) HtWTcyt c552, (B) Ht M61A, and (C) Ht M61H upon PGE electrodes. Raw data are shown, and the nonfaradaic portion of the current was subtracted, resulting in the data shown inset into each panel. All data were collected in 50 mM phosphate/citrate buffer pH 6.0, 200 mM NaCl, 0 °C, and scan rate =200 mV/s.
Figure 2.4 Cyclic voltammograms of HT M61A on 6-mercaptohexanol modified gold electrode in the absence (Black) and presence (Red) of 5 mM imidazole. All data was collected in 50 mM phosphate/citrate buffer pH 6.04, 0 °C, and scan rate = 200 mV/s.

Ligand Binding. Upon the addition of imidazole (Im) to the cell solution a modulation of the peak height of the Ht cyt c voltammetric response was observed. The normal form decreased with a concurrent increase of a lower potential state, presumed to be an Im-bound form (Figure 2.5A). Midpoint potentials for the native and Im-bound forms are summarized in Table 1. At each concentration of ligand, approximately one minute was required for the system to come to equilibrium, and multiple cyclic voltammograms were collected until a stable signal was maintained. Ht M61A was also found to be sensitive to imidazole. While the ligand-free from of the mutant was found to have a reduction potential of -163 mV, Im adduct formation began at low (μM) concentrations of the ligand, and full conversion of the electrochemical response was
attained at 5 mM Im, with a potential of -69 mV for the Ht M61A Im-complex (Figure 2.5B).
Table 2.1 Midpoint Potentials determined for the normal and imidazole bound features of Ht cytochrome c.

<table>
<thead>
<tr>
<th>Protein</th>
<th>E_m vs SHE (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal(^a)</td>
</tr>
<tr>
<td>HT WT</td>
<td>+210 ± 2</td>
</tr>
<tr>
<td>HT M61H</td>
<td>-103 ± 2</td>
</tr>
<tr>
<td>HT M61A</td>
<td>-163 ± 2</td>
</tr>
</tbody>
</table>

\(^a\)The normal, high potential couple, observed in the absence of non-native coordinating ligand. \(^b\)The low potential feature populated by the addition of imidazole. \(^c\)These additionally correspond to the reduction potentials of the normal and “met-loss” forms of Ht WT in the absence of imidazole.

Ht M61H has no change in peak position under any of the imidazole concentrations used in this study (Figure 2.5C). Imidazole binding was achieved in a concentration dependent manner for the WT and M61A mutants, and representative data of the increases in the voltammetric response due to adduct formation, and concomitant decreases in the ligand-free state, are illustrated in Figure 2.6. Binding constants for Im were estimated to be 417 ± 40 M\(^{-1}\) and 1323 ± 33 M\(^{-1}\) for Ht Wt (Figure 2.7) and M61A respectively. The binding of imidazole to cyt c films was found to be essentially kinetically irreversible on the time-scale of all electrochemical analyses. Electrodes used during the titration experiments were maintained in fresh electrochemical buffer, which was changed on a daily basis. Over the course of 5 days there was no change in the CV that could be attributed to a chemical change, while a small amount (less than 20%) of film loss was observed.
Figure 2.6 A) Cyclic voltammograms of HT WT from 0 – 100 mM imidazole. All data was collected in 50 mM phosphate/citrate buffer pH 6.0, 200 mM NaCl, 0 °C, and scan rate = 200 mV/s. (B) Change in the cathodic current intensity of the Normal (Black) and Met-loss (Red) form of HT WT as a function of imidazole concentration. Anodic current (not shown) behaves in a similar manner.
Figure 2.7 Plot of the reciprocal change in peak current as a function of the reciprocal ligand concentration for the binding of imidazole to Ht cyt c. Used to determine the association constant $K_a$. All data were collected in 50 mM phosphate/citrate buffer pH 6.04, 0 °C, and scan rate = 200mV/s.

**Redox thermodynamics.** Variable temperature PFV was conducted to determine the thermodynamic parameters, $\Delta H_{rc}^{\circ}$ and $\Delta S_{rc}^{\circ}$, that contribute to the free energy of the redox reaction; these data are summarized in Table 2.2. Over the temperature regime studied (0 – 65°C) the $E_m$ follows a linear trend, where the observed reduction potentials of the Ht WT protein ($E_m + 210$ mV at 0 °C) steadily decreased as the temperature increased. However, the potential of the “Met-loss” couple moves in a more positive direction as a function of increasing temperature. As noted above, both features of the Ht cyt c cyclic voltammogram become more reversible (peak separation decreases) as the temperature increases (Figure 2.8), and both WT proteins and M61H and M61A mutants showed little film loss until approximately 50°C, where film stability decreased.
Table 2.2 Thermodynamic parameters determined for the native and mutant forms of Ht cytochrome c determined by variable temperature PFV.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\Delta S^\circ'_\text{rc}$</th>
<th>$\Delta H^\circ'_\text{rc}$</th>
<th>$\Delta S^\circ'_\text{ML}$</th>
<th>$\Delta H^\circ'_\text{ML}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>-49.1</td>
<td>-42.2</td>
<td>95.7</td>
<td>36.7</td>
</tr>
<tr>
<td>M61H</td>
<td>-127.0</td>
<td>-31.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M61A</td>
<td>-56.2</td>
<td>-3.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*aThe average errors for $\Delta H^\circ'$ and $\Delta S^\circ'$ are $\pm 3$ kJ mol$^{-1}$ and $\pm 6$ J K$^{-1}$mol$^{-1}$ respectively.*  
*bMet-loss form.*

Figure 2.8 Variable temperature PFV response of HT WT. The initial films were generated upon PGE electrodes at 276 K (black). Subsequently the temperature was warmed to 278 (brown), 288 (blue), 298 (purple), 308 (dark green), 318 (light green), 328 (red), and 338 K (pink). All data was collected in 50 mM MOPS pH 7.0.
The temperature dependence of the reduction potential for Ht M61H and M61A are plotted along with those of native Ht cyt c in Figure 2.8 and Figure 2.9 shows the corresponding Gibbs Helmholtz plot. In every case, the data follow a linear change in reduction potential over the range studied, indicating that just one protein state is associated with the redox reactions studied at each couple. However, as the temperature was increased for the WT Ht protein, the Met-loss feature became more pronounced in terms of overall current, while the normal form decreased significantly. As the total area of each peak is equal to the total charge passed, and therefore the total population of the species engaged in a redox process occurring at that potential, either the electrochemical area or peak height can be used to calculate the relative amounts of each redox-active population. From those data, one can find the equilibrium constant ($K_{eq}$) for the

![Figure 2.9](image)

**Figure 2.9 $E_m$ vs T plot for HT WT (■) normal and (□) Met-loss, (•) HT M61A, and (†) HT M61H. Data is fit to a linear regression model where the slope = $\Delta S/nF$, where $n = 1e^-$, and shown in the solid black lines.**
Figure 2.10 Gibb-Helmoltz plot of HT WT (■) normal and (○) Met-loss forms, ( □) HT M61A, and (♦) HT M61H. Linear regression analysis was used to fit each data set and extract the redox enthalpy. Fits are shown in solid black lines.

conformational inter-conversion between the normal Met-bound and Met-loss states, as described in further detail below.

2.4 Discussion

Previously we have reported unusual, low potential redox couples for bacterial cyts c adsorbed upon carbon-based electrodes, where the low-potential form was attributed to a so-called “Met-loss” state, which may be similar to the mitochondrial alkaline transition (57). The alkaline transition is a phenomenon that has been examined primarily for mitochondrial cytochrome c and a large downward shift in redox potential is a key consequence of the alkaline conformational rearrangement (80). Interestingly, neither Ht nor Pa cytochromes contain lysine residues within loop 3, the location that has been ascribed to nitrogenous bases that are poised to replace the axial Met in adopting the
alkaline conformation. Further, generality of possible ligand displacement has for hemoproteins at PGE electrodes has been shown (22). In comparison, the refolding of Pa cyt c studied by stopped-flow kinetics additionally shows no evidence for a nitrogenous ligand rearrangement (107), while horse heart cytochrome c (Hh cyt c) goes through at least two intermediate steps in the folding process where one is a bis-His coordinated species (62). In order to elucidate the nature of the Met-loss feature (57), we have examined mutations of the axial ligand methionine from Ht cyt c, and the impact on electrochemical potential and redox thermodynamics.

**Site-Directed Mutants at Met61.** Our initial probe of the low potential feature was the installation of histidine in the axial position, where we hoped to create a stable six coordinate heme. Therefore, in correspondence to the bis-His ligated cytochromes c (108, 109), it was not surprising that Ht M61H had a single electrochemical feature at a potential significantly lower than WT protein (Figure 2.3B). The assumed bis-His heme proved to be a reasonable facsimile to the native Met-loss feature, *i.e.*, within 7 mV. Similarly, installation of Ala at the Met61 position was designed to yield an open coordination site where water/hydroxide (depending on the pH), or an exogenous ligand could bind. Investigation of the Ht M61A mutant yielded similar results as M61H, however the single couple was significantly lower in redox potential (Figure 2.3C and Table 2.1). Interestingly, these low potential species are still higher than those observed for the alkaline conformer of yeast iso-1-cyt c (80, 84).
Greater solvation of the heme pocket will likely contribute to the lowered midpoint potentials. Solvation effects have been correlated by Gray and Tezcan to account for the redox potential shifts due to heme encapsulation, where heme potentials can be tuned over a nearly 700mV window (5). For structurally characterized $c$-type hemoproteins, the increase of solvent exposure significantly decreased the observed redox potential of the heme center. Here, the M61A mutation was designed to increase solvent access, and the reduction in $E_m$ is severe (a reduction of roughly 300 mV with respect to wild-type); here the lowering of potential may not be as great as that observed by Gray and Tezcan due to interactions between the position-61 bearing loop and other structural elements. In contrast, the more negative reduction potential of Ht M61H compared to wild-type is likely the result of the new His sixth ligand donating a nitrogenous base (110). Again there are no other potential ligand candidates within the Met-donating loop, and no other His residues exist within the protein except the distal heme ligand, His16.

*Probing Met-loss Dynamics with Im binding.* Exogenous ligand binding experiments have been used extensively to probe the electronic and physical characteristics of heme proteins (111). The transient dissociation of the significantly weaker Met-Fe bond (as compared to either His or small molecules) allows for the binding of other peptide-based ligands or exogenous ligands such as imidazole, azide, cyanide, and pyridine, which will bind to the heme iron at the open sixth site of the heme (68-72, 87). The resulting ligand bound structures are not generally physiologically
relevant, but provide information on local structure and dynamics of the heme environment. Previous ligand binding studies with mitochondrial sources showed that the strongest \( \pi \)-acceptors were also ones that preferred binding to Fe(II) \((50, 70)\). This generally supports the model that ferrous iron delocalizes 3d electrons into the \( \pi \)-orbitals of soft ligands, enhancing stability of Met-Fe(II) versus Fe(III) \((70)\). To further probe the relative accessibility of the heme pocket and lability of the axial ligation, we have employed Im-binding as a means to reveal, remove, and replace the labile Met61 ligand. Previously we have demonstrated that Pa cyt \( c \) can bind Im when adsorbed at electrodes \((57)\). Figure 2.5 demonstrates that native Ht cytochrome and Ht M61A, have similar susceptibilities to form imidazole adducts that replace the axially coordinating ligand, while Ht M61H does not provide any electrochemical evidence for adduct formation. Imidazole is a good mimic for histidine ligation, which is supported by the structural analysis of Hh cyt \( c \) \((74)\). Formation of Ht cyt \( c \)-Im adducts for either the WT protein or M61A produce species that are electrochemically very similar, although not identical. Modulation of the peak intensities \( (i_p) \) for both forms, native and imidazole-bound, occurs in a concentration dependent manner (Figure 2.6). The similar nature of the redox potentials, support the idea that Im-binding and subsequent structural rearrangements tune the reduction potential coarsely, while secondary coordination sphere interactions \( (e.g., \text{hydrogen bonding and solvent interactions}) \) further refine the midpoint that is observed \((50, 78, 79)\). The effects of the ligand binding are not only seen in the full length protein but also in models such as microperoxidases, the 6 to 11 amino acid long
proteolytic products of mitochondrial cytochromes (54, 112). Binding of nitrogenous ligands to N-acetyl-microperoxidase-11 (AcMP11) decreases the overall reduction potential with respect to the water bound form. Furthermore the bulkier ligands result in the more pronounced decreases in reduction potential (78). The association constants, $K_a$, for the Im adduct of both Ht and M61A are orders of magnitude greater than that observed for Hh cyt c (106). Yet they are of a similar magnitude to those observed for Rhodobacter sphaeroides (Rs) and Rhodobacter capsulatus (Rc) cytochrome $c_2$ (75, 86), which contain additional “hinge-like” regions adjacent to the Met-bearing loop absent in either Ht or Hh proteins. Perhaps more significant, the data collected here are all from an adsorbed mode, while prior efforts have monitored diffusion-based associations of Im and the cyt c of interest. Thus, while Ht appears to have an enhanced affinity for Im with respect to the Hh protein, this trait may be a reflection of the surface interactions between the protein scaffold and PGE, which result in an enhanced stability of the Met-loss state, and loop rearrangements that are required in that process.

Redox Thermodynamics and the Met-loss Equilibrium. We examined the redox thermodynamics of Ht cyt c and its Met61 mutants in order to understand the effects of our mutations on the fundamental driving force for the electron transfer process. Figure 2.8 depicts the typical electrochemical response with the change in temperature, resulting in redox thermodynamic parameters summarized in Table 2.2. Overall these values correspond similarly those described by both Sola and Gray for mitochondrial cytochromes, as well as heme microperoxidases (78, 91, 98, 99). A comparison of the
entropic and enthalpic terms (Figure 2.9 and Figure 2.10, Table 2.2), for the native and Met-loss conformers of Ht cyt c, indicate that while sign of $\Delta S^\circ_{rc}$ has switched, the difference between redox potentials is still largely driven by differences in enthalpy, where the enthalpic contributions have been correlated to stabilization of the Fe(II) state(50, 78). Some small curvature was noted during the analysis of the alkaline form, however these are within error of the measurements. In several mitochondrial cases, clear linear breaks have been reported in the entropic and Gibbs-Helmholtz plots (97). An interesting observation is the entropic loss for M61H, which displays a largest entropic contribution of the proteins studied here, and with a negative magnitude, suggestive of the decreases in $\Delta S^\circ$ that have been observed for aqueous metal ions (M$^{2+/3+}$) and attributed to shielding by large organic ligands in aqueous environment (113, 114). Entropic/enthalpic compensative forces due to solvation are consistent here as well (115). Notably, our data are in good general agreement with the magnitude of redox thermodynamic parameters measured by voltammetry utilizing semipermeable membranes for native HT, and mutations affecting electrostatic interactions of the N- and C- termini (100, 116, 117). The comparison indicates that the M61 mutations have not overwhelmingly affected the intrinsic thermodynamics associated with the electron transfer for Ht cyt c.

An unexpected benefit from the temperature dependence of the Ht cyt c electrochemical data is observation of variable peak current intensity ($i_p$) of the normal and Met-loss forms over the temperature range investigated. As Figure 2.8 shows, the
area and $i_p$ for the two forms appear to be inversely correlated: as the temperature increases the percentage of normally folded Ht cyt $c$ diminishes while the Met-loss form accumulates. We found these changes to be fully reversible by jumping the temperature, and that the population intensities rapidly equilibrated, ensuring that at each temperature the system was equilibrium. Additionally, we note that the equilibrium surface concentration of the two species cannot be due to pH-induced effects: as we have shown previously for the WT Ht protein, at pH 6.0, the mid-point potential is invariant as a function of pH. Thus, we could quantitatively assess the surface concentration of both states as a function of temperature, by using either $i_p$ or the area of the electrochemical signature, and thereby we determined the equilibrium constant associated with the thermally driven conversion of the normal form to the Met-loss state ($79, 91, 92$). In this way, we have been able to elaborate upon protein electrochemistry as a tool to examine the free energy associated with conformational changes ascribed to groups of residues that cooperatively interact, termed foldons, joining the ranks of hydrogen exchange mass spectrometry and NMR, which are more widely used to interrogate foldon thermodynamics ($118$). The free energy of the alkaline transition ($\Delta G_{\text{AT}}$) has been investigated electrochemically through the thermodynamic parameters ($\Delta S_{\text{alk}}$ and $\Delta H_{\text{alk}}$) as a function of an apparent equilibrium constant ($K_{\text{app}}$) ($91$). And here, we can directly measure the normal and Met-loss populations as a function of temperature, using either the total current passed associated with the individual redox couples (peak area) or simply the maximal height of the current response ($i_p$). Both of these attributes were used
to calculate the equilibrium constant describing the conversion from normal form to the Met-loss state, \( K_{ML} \). And a subsequent plot of \( \ln (K_{ML}) \) vs. \( T^{-1} \) (Figure 2.11) yields a straight line with slope of \( -\Delta H^\circ / R \) and y-intercept \( \Delta S^\circ / R \). The enthalpy for the conversion to the Met-loss state is 37.0 ± 0.2 kJ mol\(^{-1}\), a value that is on par with the sub-global free energies of hydrogen exchange (\( \Delta G_{HX} \)) and those determined for \( \Delta G_{AT} \) from direct voltammetry\( (79, 92, 98, 118-124) \). However, utilizing the classic assumption that the entropic term remains independent of temperature, we find that the Gibbs free energy (\( \Delta G_{ML} \)) for the Met61 loss is 6.3 ± 0.1 kJ mol\(^{-1}\) at 273 K, indicating that entropy contributes substantially to the overall free energy of the conversion.

![Figure 2.11](image_url)

**Figure 2.11** Plot of \( \ln(K_{eq}) \) vs 1/T used to determine the Gibbs free energy of Met61 loss. The equilibrium constant \( (K_{eq}) \) was determined by (●) peak area and (▼) peak intensity. Linear regression analysis was used to fit each data set and are shown by the solid line.

While it is unclear whether the assumption of constant entropy and enthalpy over all temperatures is appropriate, Table 2.3 places these data in the context of free energies associated with other cytochrome c folding substructures, or foldons, as determined by
hydrogen/deuterium exchange ($\Delta G_{HX}$) or for the ($\Delta G_{AT}$). Currently the foldon assignment of Ht cyt c$_{552}$ is not available, however we note that the Met61-donating loop of the corresponding Hh and Pa proteins have very similar $\Delta G_{HX}$ values. While it is possible that our use of a van’t Hoff plot to determine $\Delta G_{ML}$ has over-estimated the entropic contribution to the free energy, the enthalpic term is in excellent agreement with the energetics associated with localized unfolding for other cyts c. Yet the apparent overall lowering of free energy for loss of the Met ligand, when compared to the foldon energies measured for either Pa or Hh proteins or the free energy for the alkaline transition for YCC(92) or its mutants (79) (monitored by diffusional voltammetry at passivated gold electrodes), again likely reflect the Table 2.3. Comparison of Gibbs free energy for the Met-loss form (ML), sub-global hydrogen exchange of the methionine donating loop (HX), and mitochondrial alkaline form (AT). difference in monitoring localized unfolding at a surface, versus free in solution. Indeed, it appears that PGE itself lowers the free energy requirements for loss of the Met ligand. Again we can turn to the structural models for the local ligand dynamics in cyts c to provide some insight into the Met-loss state we observe here. In the case of the Rc and Rs cyts c$_2$, Dumortier and Cusanovich provide evidence that a local region flanking the axial ligand (Met 96, Rc numbering) facilitate different aspects of the isomeration (75, 85-87). Amino acid residues 93 and 95 in the Met-bearing loop assist the conversion of the closed to open form while residues 98 and 99 affect the imidazole affinity by providing better solvent access (86).
Table 2.3 Comparison of Gibbs free energy for the Met-loss form (ML), sub-global hydrogen exchange of the methionine donating loop (HX), and mitochondrial alkaline form (AT).

<table>
<thead>
<tr>
<th>Species</th>
<th>$\Delta G_{\text{ML},273,K}$</th>
<th>$\Delta G_{\text{HX}}$</th>
<th>$\Delta G_{\text{AT}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H_{t}\text{ cyt }c_{552}^{a}$</td>
<td>6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Pa\text{ cyt }c_{551}^{b}$</td>
<td>26 - 34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Hh\text{ cyt }c^{c}$</td>
<td>26.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef heart cyt $c^{d}$</td>
<td>52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YCC$^{e}$</td>
<td>48</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Error for both the upper and lower bounds are ± 0.2 KJ mol$^{-1}$; $^b$Ref. 56; $^c$Ref. 123; $^d$Ref. 91; $^e$Ref. 92.

Thus spanning the entire region of residues 88-102, the behavior is akin to a “hinge” (75, 85-87). Yet, the first NMR model of the YCC alkaline form from Gray, Mauk, Bertini and coworkers demonstrated that rearrangement of the Met-donating loop is drastic, where there is a general increase in loop mobility, and an absence of other protein contacts aside from Lys73-Fe ligation (73). Imidazole binding to horse heart cyt $c$ similarly goes through substantial backbone reorganization for the residues around Met80 (the equivalent to Met61 in the bacterial proteins) to accommodate the new small molecule ligand. NMR models show that the plane of the bound imidazole is perpendicular to the distal histidine, and acts similar to a histidine ligand with respect to orientating the heme the magnetic properties (74). These exemplify a key element of the Met-Fe coordination: the local structural dynamics play a crucial role in the maintenance of the coordination. In contrast, temperature dependent NMR and optical spectroscopy
studies of bacterial cyt c orthologs (members of the cyt c₈, such as the Ht protein studied here) have been used to support a model where the maintenance of the Met-Fe(III) is linked to global, rather than local stability (93, 95). Thus, by using direct electrochemical analyses, we have demonstrated a previously underappreciated similarity between bacterial cyts c₈, mitochondrial, and cyts c₂. And bridged a gap in our understanding of the importance of localized unfolding in the stability of the Met-Fe(III) bond. The Met-loss form can be additionally informed by comparison with the *Bacillus pasteurii* cyt c (Bp cyt c) to the Ht protein. In the Bp cytochrome, the Met-Fe interaction is extremely stable under a vast pH range, and upon the loss of the axial ligation, the protein totally unfolds (125). In terms of sequence, the Met-bearing loop of the Bp protein is 9 amino acids in length and rich in Gly and Pro residues, a combination of factors that yield a very rigid structure (125). The Met-donating loop found in the Ht cytochrome is also rich in Gly and Pro: it contains four Gly residues throughout the first half of the loop, and three Pro residues, which flank the axial Met. However, the loop is larger, 17 amino acids, and the increases size may decrease the overall energy barrier for the isomerization required for the Met-loss process studied here (61).

The question remains: following the Met-loss process, what is the nature of the new ligand, if any? Notably, the data presented here for the M61A mutant indicate that the Met loss state likely includes a protein-based ligand in the distal position. In terms of potential ligands, there are no other His present in the Ht cyt c sequence, but the closest available lysine residues (K49 and K50) are homologous to K72/K73 of mitochondrial
cyts $c$ (where K73 is known to participate in formation of the alkaline conformer along with K79 (28-30), which is not a conserved lysine in the Ht protein). K49 and K50 are at the terminus of helix three, where they participate in hydrogen bonding (61), while helix three also has residues that contribute to the hydrophobic core of the protein, playing a key role in protein folding (61, 124). In the homologous Pa cyt $c$ helix three and the Met-donating loop are known to exist within the same foldon, displaying a free energy of unfolding of 26-34 kJ mol$^{-1}$ (124). While identical data is not available for Ht, the lower free energy reported here may represent the unfolding and ligand rearrangement of a smaller element of structure, or an overall decrease of the necessary driving force, due to the interaction with the electrode. Either way, similarities in structure suggest that partial unfolding of helix three may not totally perturb the whole structure due to normal solution dynamics (118). Thus, we hypothesize that the $\Delta G_{\text{ML}}$ for the Met-loss transformation reported here may be concomitant with localized helix fraying, in which one of the terminal Lys residues of helix three binds at the newly available coordination site, in a manner that is exchangeable with Im. Here we have shown that large concentrations of imidazole are required to fully remove the native coordination, and the imidazole bound form and Met-loss form have similar electrochemical identities. Validating this hypothesis with electrochemically-poised spectroscopies will be an ongoing research effort in the future.
2.5 Conclusions

In conclusion, we have begun to elucidate the nature of the Met-loss feature exhibited by Ht cyt $c_{552}$ on PGE electrodes. We have shown that the Ht cyt $c$ is susceptible to ligand replacement by the small molecule imidazole. The imidazole-cyt $c$ adduct is similar to the low potential Met-loss feature initially associated with PGE surface. And similarly mutations to Met61 can be made to mimic that of the low potential form in the presence of an imidazole ligand (His based or extrinsic). Redox thermodynamics associated with the normal and alkaline forms of WT, M61A, and M61H Ht cytochromes are consistent with those from other species. Finally the required driving force for reorganization of Ht cyt $c$ Met-bearing loop is lowered by the PGE surface, displaying an equilibrium that can be readily enhanced at modest, yet elevated temperatures. The conformational change may be physiological, however the impact may be dictated by the local versus global stability associated with the Met-donating loops.
Chapter 3 Methionine ligand lability of homologous cytochromes c

3.1 Introduction

In Chapter 2 we interrogated the Met-loss feature observed in the model system Ht cyt \( c_{552} \) as a function of electrode material. Ht cyt \( c_{552} \) is susceptible to axial ligand replacement by exogenous small molecules such as imidazole, and the Im-adduct is similar in reduction potential to the Met-loss feature. Mutation of the axial Met to His can be made to mimic the Met-loss feature. The redox thermodynamics associated with the normal and Met-loss species is consistent with those from other species. Finally, interaction with the PGE surface substantially lowers the free energy for the reorganization of the Met-donating loop, which can be enhanced with modest increases in temperature \( (126) \). The work in this chapter builds upon our previous findings to characterize the phenomenological Met-loss of bacterial monoheme cytochromes \( c \) as a function of electrode material.

Analysis of homologous proteins is an appealing strategy to determine how variations in amino-acid sequence and structures translate into differences in function. Evolutionary pressures may be efficient such that small changes in sequence can translate into substantial modifications of stability, conformation, and function. Here, our interests in the sequence-structure-function paradigm are not to the point of de novo design \( (127) \). Instead, we have chosen a series of homologs of the Ht cyt \( c_{552} \) in Chapter 2, and examine them by analogous PFV-based redox-induced folding analysis and extend our analysis to thermal melting visualized via circular dichroism (CD).
Among bacterial class I cytochromes c there is a wide variation in redox potential despite similar protein scaffolds. Several mechanisms are implemented to modulate the electronic properties of the heme such as axial ligand and its orientation, ruffling of the porphyrin ring, and second sphere interactions \( (55, 128) \). Ht cyt \( c_{552} \) discussed in Chapter 2 is a class I cytochrome c, and is also member of the cytochrome \( c_8 \) structural family; additional members include cyts \( c \) from \textit{Pseudomonas aeruginosa} (Pa) and \textit{Nitrosomonas europaea} (Ne). Sequence alignments are shown in Figure 3.1.

![Sequence alignment of monoheme cytochromes c from \textit{Hydrogenobacter thermophilus} (Ht), \textit{Pseudomonas aeruginosa} (Pa), \textit{Nitrosomonas europaea} (Ne) and \textit{Shewanella oneidensis} (So). The alignment was carried out with ClustalW2.](image)

Ht and Pa cyt \( c_{551} \) have historically served as a complementary thermophile/mesophile pair for studying folding/stability \( (59) \). Comparative studies have identified key residues that influence the heme electronic properties, and ultimately the redox potential \( (59, 65, 129) \). Ht, Pa, and Ne cyts \( c \) are remarkably similar in sequence and structure (Table 3.1), yet their redox potential and pH dependent properties are remarkably different \( (57, 129) \). Among the cyt \( c_8 \) family, residue 64 (Pa numbering) is a Asn residue that provides a hydrogen bonding interaction to the \( \delta S \) of the axial Met, stabilizing the side chain conformation with respect to the axial His orientation. In the case of Ht, that hydrogen
bonding interaction is no longer present due to the substitution of a Gln, allowing for the rapid conversion between R and S conformations (Figure 3.2), or “fluxional” (64-66, 129).

<table>
<thead>
<tr>
<th></th>
<th>Ht</th>
<th>Ne</th>
<th>Pa</th>
<th>So</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ht</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ne</td>
<td>2.013</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pa</td>
<td>0.471</td>
<td>2.055</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>So</td>
<td>2.884</td>
<td>3.233</td>
<td>7.575</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.1 Root mean squared deviation (RMSD) in angstroms of the carbon backbone of homologous cytochromes c from Hydrogenobacter thermophilus (Ht, PDB ID 1YNR), Pseudomonas aeruginosa (Pa, PDB ID 451C), Nitrosomonas europaea (Ne, PDB ID 1A56), and Shewanella oneidensis (So, PDB ID 1XK7). Alignments were done with PyMol.

Ne cyt c_{551} is also fluxional with respect to the axial Met conformation, and is about 60% identical to Ht. Val insertion at the 64 position disrupts the internal hydrogen bonding; deletion of Val64 (Ne V64Δ) allows for the reinstatement of the hydrogen bonding network with the axial Met, and establishes a single Met61 side chain conformation ultimately affecting the redox potential (129).

Figure 3.2 Conformational flexibility of a fluxional Met ligand. Met Cε over pyrrole I is in the R conformation and representative of mitochondrial cyts c. Met Cε over pyrrole IV is in the S conformation and representative of Pa cyt c. Ht and Ne have been shown to rapidly interconvert between R and S conformations. P indicates propionate.
In this chapter we also extend our studies of the relationship between folding, ligand rearrangement, and redox properties of the cytochrome c₈ family to an ortholog from *Shewanella oneidensis* (So), the most divergent member of the class 1 cytochrome c considered here (So ScyA). Native purification of So ScyA was done by Meyer and coworkers (130). The overall abundance under several growth conditions suggests that it serves as an electron mediator in the periplasmic space. So ScyA when adsorbed onto PGE electrodes displays the canonical normal form at high redox potentials and Met-loss feature approximately -100 mV vs. SHE. Sequence identity of So ScyA compared to members of the cyt c₈ family is shown in Table 3.2.

<table>
<thead>
<tr>
<th></th>
<th>Ht</th>
<th>Pa</th>
<th>Ne</th>
<th>So</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ht</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pa</td>
<td>56</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ne</td>
<td>60</td>
<td>54</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>So</td>
<td>6</td>
<td>13</td>
<td>18</td>
<td>100</td>
</tr>
</tbody>
</table>

The So ScyA cytochrome configuration has not been formally reported as fluxional, however the initial report by Bertini and Bartalesi (131) indicated that the proton (¹H) hyperfine shifts signals are not spread as far downfield as those of other members of the family. Proton reasonances can range downfield > 80 ppm, and the compression of these signals is indicative of a fast equilibrium on the NMR time scale. The 1D ¹H NMR resonances broaden with an increase in temperature, also indicating multiple species. Similar phenomena have been reported by Bren et. al. for members of
the fluxional members of cytochrome \( c_8 \) family. Examination of the solution structure reveals no analogous residue to Asn 64 in Pa that could enforce a single Met conformation.

Here we investigate the ligand binding and redox thermodynamics of three homologous cytochromes c in order to further elucidate the nature of the Met-loss form associated with bacterial monoheme cytochromes \( c \) on PGE electrodes. Replacement of the axial Met with exogenous ligands will allow us to compare the adduct redox potential with those of other cytochromes and model systems. Investigation of the redox thermodynamics will also allow us to compare the principal components of the electrochemical driving force for normal and Met-loss states. Our results correlate well with other cytochromes \( c \). Finally, we are able to compare the relative populations of the normal and Met-loss states on the electrode to elucidate the Gibbs free energy for the conformational change.

3.2 Materials and Methods

3.2.1 Expression and Purification of \( S. oneidensis \) ScyA.

\( S. oneidensis \) ScyA (SO0264) was synthesized by GenScript USA using codon optimization for \( E. coli \) K-12 (Figure 3.3). There were two vector targets during the synthetic process. The EcoRV site was utilized to initially incorporate the synthetic product into pUC57, an ampicillin resistant (amp) vector with the M15 origin. The pET25b+ (Novagen) vector was chosen as an expression vector and \( S. oneidensis \) ScyA was inserted between the NdeI and BamHI restriction sites.
Recombinant So ScyA was expressed in *E. coli* JM109 bearing the pEC86 plasmid. 10 mL pre-cultures were grown overnight in 2xYT medium. Large cultures were inoculated with a 1:100 dilution and incubated at 37 °C, 200 rpm, for 22 hours. Overexpression was achieved through auto induction (REF). All media was supplemented with 100 ug/mL ampicillin and 34 ug/mL chloramphenicol.

Cell pellets were harvested by centrifugation (6000 x g, 15 min, 4 °C), resuspended in 10 ml/g 20 mM Tris 1 mM EDTA, 50 mM NaCl, pH 8.0 and 0.1 mg/mL lysozyme, and lysed by sonication. Cell debris was removed by centrifugation. Clarified lysate was adjusted to pH 4.5 by dropwise addition of 1 M acetic acid, and precipitated proteins were removed by centrifugation. The resulting pink supernatant was oxidized by addition potassium ferricyanide. Care must be taken during the oxidation step, too gross an excess of oxidant will cause the So ScyA to precipitate. Oxidized So ScyA was loaded onto MacroPrep High S resin (Bio-Rad) equilibrated in 10 mM sodium acetate pH 4.5. So ScyA eluted over a gradient from 50-500mM NaCl. Eluted fractions around 250 mM NaCl visualize on SDS page gels, showing a single band corresponding to ~8.5 KDa (calculated MW 8361.51 Da). Fractions with purity ratios (A410/A280) of ≥ 4.0 were pooled and stored in 15% glycerol at −80 °C.
3.2.2 Production of Pseudomonas aeruginosa cytochrome c

Recombinant Pa cyt c were expressed and purified in the laboratory of Professor Kara L. Bren at University of Rochester as described previously (134). Briefly, the purification of Pa cyt c551 was conducted by first loading the cleared lysate onto DEAE resin (GE Healthcare) equilibrated with 10 mM Tris-HCl pH 7.5. Protein fractions were collected over a 10-40 mM Tris-HCl gradient and concentrated prior to the next purification step. The pH was lowered to below pH 4.0 with acetic acid before loaded onto a CM column (GE Healthcare) equilibrated with 10 mM sodium acetate buffer, pH 4.0. The protein is eluted in 10 mM sodium acetate using a pH gradient between 4.0 and 6.0.

3.2.3 Expression and Purification of N. europaea Cytochrome c

The plasmid pSNEC bearing the gene encoding Nitrosomonas europaea (Ne) cytochrome c551 was a generous gift of Dr. Kara L. Bren at University of Rochester. pSNEC (AmpR) was transformed into JM109 E. coli harboring the pEC86 (CMR) containing the cytochrome c maturation cassette. Pre-cultures (5 mL) were grown overnight in 2xYT medium. Large cultures were inoculated with a 1:1000 dilution of the pre-culture and grown for 22 hours, 175 rpm, at 37 °C. Cells were harvested by centrifugation (6000 x g, 15 min, 4 °C) and resuspended in 10 in 10 mM Tris-HCl, pH 7.0. Cells were disrupted by sonication and cell debris removed by centrifugation (10000xg, 15 min, 4 °C). The cleared supernatant was adjusted to pH 4.6 with 1M acetic acid and loaded onto CM resin (GE Healthcare) equilibrated with 10 mM sodium acetate,
pH 4.5. Ne cyt c₅₅₁ was eluted with a 0-180 mM NaCl gradient. The pink fractions were collected, concentrated, and oxidized using potassium ferricyanide. Similar to So ScyA, a gross excess of oxidant causes the protein of interest to precipitate. The oxidant was removed using a PD-10 column (GE Healthcare) and was loaded onto MacroPrep High S resin (Bio-Rad) equilibrated in 10 mM sodium acetate pH 4.5. The protein was eluted with a 0-180 mM NaCl gradient.

3.2.4 Electrochemical Methods.

All PFV measurements were carried out on a PGSTAT30 or PGSTAT12 (Ecochemie) electrochemical analyzer equipped with FRA and ECD modules. A three electrode configuration was used in a water-jacketed electrochemical cell. A resin-body calomel electrode (Accumet) and platinum wire were used as the reference and counter electrodes respectively. The calomel reference was maintained at a constant temperature (293 ± 0.5 K). Cell temperature was maintained using a refrigerating circulator. Pyrolytic edge-plane graphite (PGE), embedded in resin served as the working electrode for redox thermodynamics and ligand binding studies. PGE electrodes were polished with an aqueous 1μ alumina slurry, followed by sonication. Protein films were generated by directly applying 2 μL cytochrome c. Excess protein was washed away with cold buffer. pH-dependent voltammetry was conducted with alkanethiol modified gold electrodes. Gold electrodes were sanded, then polished with alumina (1.0, 0.3, 0.05 μM, Buehler), with sonication between each grit to remove dust and excess polish. The last polishing step was done with a monocrystalline diamond suspension (3 μM, Buehler) and abrasive
pads (Buehler). Electrochemical cleaning was carried out in 0.1 M H₂SO₄ by cycling 0.2 – 1.35 V (vs. SCE). Cleaned gold surfaces were modified in an 2 mM ethanolic solution of 6-mercaptohexanol for 12 hours. Excess alkane thiol was removed by rinsing with ethanol followed by water and then considered ready for use. Protein films were generated on both surfaces by directly applying 2 μL cytochrome c. Excess protein was washed away with a small amount of cold buffer.

The redox thermodynamics were examined by protein film voltammetry (PFV) as previously described (126) and in Chapter 2.

3.2.5 Circular dichroism and thermal denaturation

Circular dichroism (CD) spectroscopy was performed on an Applied Photophysics CS/2 Chirascan spectrophotometer equipped with a CD 250/AP temperature-controlled cuvette holder (Quantum Northwest), and controlled with a TC125 Temperature controller (Quantum Northwest). Measurements were conducted with a 0.10 cm path length two-piece quartz cell (Starna). All experiments used 50 μM protein. CD spectra were collected with a window 180 – 280 nm, averaging time of 1s and a band width of 1 nm. Thermal unfolding was monitored as either a continuous ramp experiment at 0.5 °C min⁻¹, or as a step ramp with 2 °C steps and 2 min equilibration time at each temperature over 10 – 92 °C. Reversibility of samples without denaturant was determined by return of the temperature to 10 °C, collection of a final CD spectrum and verification that the spectrum is the same as that of the starting material.
Guanidine hydrochloride (GuHCl) was used for denaturation experiments. A fresh stock solution of GuHCl (Sigma) was used and diluted in 5 mM MES pH 7.0. The pH of each protein solution was adjusted with a small amount of sodium hydroxide to pH 7.0. The ionic strength of each sample was maintained at 1.25 M with sodium sulfate. Unfolding was monitored using a step ramping experiment as described above with GuHCl ranging from 0.25 – 1.25 M. Reversibility was determined by removing the denaturant and verifying the CD spectra of each protein sample. Spectra were collected from 180 – 280 nm as described above, however below 200 nm the spectra are distorted due to the presence of GuHCl and will only be displayed in the wavelength range of 200 – 280 nm.

Data analysis was done by determining the fraction folded (α) at any given temperature by processing the change in ellipticity at 222 nm (Equation 3.1) (135, 136).

\[
\alpha = \frac{\theta_t - \theta_F}{\theta_F - \theta_U}
\]  
(Eq 3.1)

The parameters of equation 3.1 are the observed ellipticity at a given temperature (\(\theta_t\)), for the fully folded (\(\theta_F\)), and the fully unfolded (\(\theta_U\)) proteins. The thermal unfolding temperature (\(T_m\)) is determined when \(\alpha = 0.5\). Determining the \(T_m\) at zero denaturant was done using a linear extrapolation method by plotting \(T_m\) vs. [GuHCl] (59, 135-137).

3.3 Results

This work builds upon our previous work with Ht cyt c as a model system for interrogating the structure function-relationship that impacts redox potential with respect
to “Met-loss” state (126). As in our prior work (57, 126, 129), all three cytochromes c yielded excellent electrochemical responses when adsorbed onto PGE electrodes. So, Pa, and Ne cyts c exhibited a typical reversible redox couple that is high in potential, and a consistent value for the “Met-Loss” form of approximately -100 mV.

3.3.1 Ligand binding

Upon the addition of imidazole (Im) to the cell solution, a modulation of the PFV response was observed in all cases. The increase of a low-potential species is presumed to be due to formation of an Im-adduct. Midpoint potentials of Im-adducts may be found in Table 3.3.

Table 3.3 Midpoint potentials determined for the normal and imidazole bound adducts of homologous cytochromes c from Shewanella oneidensis (So), Pseudomonas aeruginosa (Pa), and Nitrosomonas europaea (Ne) at pH 6.0.

<table>
<thead>
<tr>
<th>Species</th>
<th>$E_m$ vs SHE (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>normal</td>
</tr>
<tr>
<td>So</td>
<td>313 ± 3</td>
</tr>
<tr>
<td>Pa</td>
<td>277 ± 3</td>
</tr>
<tr>
<td>Ne</td>
<td>205 ± 2</td>
</tr>
</tbody>
</table>

Imidazole was directly added to the cell solution and incubated for one minute allowing the system to come to equilibrium. Multiple cyclic voltammograms were collected until stable voltammetric response was maintained. So, Pa, and Ne adsorption were sensitive to the addition of imidazole to the cell solution. Adduct formation began at low (µM) concentrations of Im, however full conversion was never achieved due to eventual
desorption of the protein from the electrode. Similar to the Ht system (126), once the Im-adduct was formed the release and establishment of the normal form was not observed on the time scale of the PFV experiment.

3.3.2 Redox thermodynamics

Variable temperature PFV was used to elucidate the thermodynamic parameters, $\Delta H^\circ_{rc}$ and $\Delta S^\circ_{rc}$, that contribute to the Gibbs-free energy of the redox reaction and are summarized in Table 3.4.

**Table 3.4 Thermodynamic parameters determined by protein film voltammetry for the normal and Met-loss forms of various bacterial cytochromes c.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\Delta S^\circ_{rc}$</th>
<th>$\Delta H^\circ_{rc}$</th>
<th>$\Delta S^\circ_{ML}$</th>
<th>$\Delta H^\circ_{ML}$</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ht Wt</td>
<td>-49.1</td>
<td>-42.2</td>
<td>95.7</td>
<td>36.7</td>
<td>Ch2 and 126</td>
</tr>
<tr>
<td>Ht M61H</td>
<td>-127</td>
<td>-31.1</td>
<td>95.7</td>
<td>36.7</td>
<td>Ch2 and 126</td>
</tr>
<tr>
<td>Ht M61A</td>
<td>-56.2</td>
<td>-3.7</td>
<td>57.9</td>
<td>23.6</td>
<td>Ch2 and 126</td>
</tr>
<tr>
<td>Pa</td>
<td>-28.9</td>
<td>-32.9</td>
<td>57.9</td>
<td>23.6</td>
<td>This study</td>
</tr>
<tr>
<td>Ne</td>
<td>-67.5</td>
<td>-37.16</td>
<td>19.3</td>
<td>18.4</td>
<td>This study</td>
</tr>
<tr>
<td>So</td>
<td>-57.9</td>
<td>-46.5</td>
<td>57.9</td>
<td>23.4</td>
<td>This study</td>
</tr>
</tbody>
</table>

$^a$The average errors for $\Delta S^\circ$ and $\Delta H^\circ$ are $\pm 4$ J K$^{-1}$ mol$^{-1}$ and $\pm 3$ kJ mol$^{-1}$ respectively.

$^b$Met-Loss form.

A typical electrochemical response for the variable temperature PFV of So ScyA is shown in Figure 3.4. Over the temperature regime studied (0–50 °C), the redox potential, $E_m$, follows a linear trend (Figure 3.5). The film loss over the course of the experiments was minimal at temperatures below 35 °C. At the higher temperatures, film stability was greatly diminished in comparison to Ht, Pa, and Ne adsorption. The temperature dependences of the reduction potential for both normal and “Met-loss” forms are plotted
along with the corresponding Gibbs-Helmholtz plots for So ScyA, Pa cyt $c_{551}$, and Ne cyt $c_{552}$ (Figure 3.5). In each case the change redox potential is linear with respect to

Figure 3.4 Variable temperature PFV response of So cyt c$. The initial film was generated on a PGE electrode. The film was generated at 273 K (black). Subsequently, the temperature was steadily warmed linearly to 275.5 K (blue), 278 K (light blue), 280.5 K (teal), 283 K (brown), 285.5 K (red), and 288K (pink). All data was collected in 50 mM 3-(N-Morpholino)propanesulfenic acid (MOPS), pH 7.0.
temperature suggesting that only one species exist for each redox couple. A similar phenomenological change in current intensity for Ht cyt $c_{552}$ was observed for So, Pa, and Ne cyts $c$. As the temperature was increased a loss of the normal form occurred with a concurrent increase in the “Met-loss” feature. The electrochemical area or peak current may be used to calculate the relative amounts of each redox active species on the electrode. Similar to the observation for Ht proteins discussed in Chapter 2 (126), the modulation of peak height and area are reversible when the temperature is lowered. From those data we are able to find the equilibrium constant for the conformational change ($K_{eq,ML}$) between the native Met-bound and Met-loss states.
3.3.3 pH dependence

![Graph showing pH dependence of redox potential](image)

Figure 3.6 Influence of pH upon the redox potential $E_m$ for So ScyA. Voltammograms were collected in 5 mM MES HEPES TAPS CHES, 100 mM NaCl. The slope of So ScyA is 5 mV/pH unit and therefore, pH independent. The expected slope of 1H$^+$/1e$^-$ coupled electron transfer is expected to be -58 mV/pH unit.

The influence of pH on the redox potential of So ScyA was used as an additional foil for Ht, Pa, and Ne cyt $c$; each has a redox potential that is dependent on the pH of the cell solution (129). Dependence is evaluated by the slope (s) of the plot $E_m$ vs. pH, and described by Equation 3.2 for $n$ electrons and $m$ protons.

$$\frac{2.3RT}{F} \cdot \frac{m}{n} \text{ in V/pH unit} \quad \text{(Eq. 3.2)}$$

If the reduction potential is dependent upon pH, it decreases at -59mV/pH unit (@ 25 °C). In the case of So ScyA the decrease we observe is -5 mV/pH unit. So ScyA may be considered pH independent, in contrast with the other cyts $c$ studied here that have clear proton-coupled regions (129).
3.3.4 Circular dichroism

Thermal denaturation monitored by circular dichroism was used to evaluate the relative stability of Ne cyt c and So ScyA with respect to the reported values for Pa and Ht cyt c[59]. Importantly, this allowed us to evaluate the relative stability of our system with respect to the observed Met-loss feature and conformational plasticity. Melting studies could be performed in the absence of denaturant for So ScyA. Spectra were collected in both continuous ramp and step-gradient modes. Both produced reproducible results with an average deviation in observed melting temperature of 0.3 °C. Figure 3.7 and Figure 3.8 are representative of the data collected for So ScyA and Ne cyt c. All of the Ne cyt c data was collected in the step-gradient mode which yielded reproducible results most consistently. The overall shape of the spectra are similar however the dip at 222 nm is much more pronounced for So ScyA (Figure 3.7A) than for Ne cyt c (Figure 3.8) despite their overall structure being similar (Table 3.1). The overall shape of the CD spectra are prototypical for alpha-helical proteins[136, 137].
Figure 3.7 (A) Representative CD spectra of So SycA highlighting 10 (glue), 66 (green), 92 °C (red), (B) and extracted ellipticity at 222 nm for 50 µM So ScyA from 10 – 92 °C. 5 mM MES 10 mM NaCl pH 7.0. Collected in continuous ramp mode 0.2 °C/min with 1 s averaging, and 1 nm bandwidth.

Figure 3.8 Representative CD spectra of Ne cyt c in the presence of 1.25 M GuHCl highlighting 10 (glue), 54 (green), 92 °C (red), (B) and extracted ellipticity at 222 nm for 50 µM So ScyA from 10 – 92 °C. 5 mM MES 10 mM NaCl pH 7.0. Collected in continuous ramp mode 0.2 °C/min with 1 s averaging, and 1 nm bandwidth.
Ne cyt c was too stable to acquire a full melting curve in the absence of denaturant. Here we have repeated the melting experiment in the presence of varying amounts of GuHCl (Figure 3.9), after which we extrapolated to zero GuHCl using the linear extrapolation method (Figure 3.10) (137).

![Figure 3.9 Thermal unfolding curves for Ne cyt c in the presence of \(\odot\) 1.25 M, \(\triangle\) 1 M, \(\bullet\) 0.75 M, and \(\blacksquare\) 0.5 M GuHCl. Samples were in 5 mM MES pH 7.0 with GuHCl and \(\text{Na}_2\text{SO}_4\) to make the total ionic strength 1.25 M. Collected in step ramp mode 2 °C steps with 2 minute equilibration time, 1 s averaging, and 1 nm bandwidth.](image1)

![Figure 3.10 Plots of \(T_m\) as a function of [GuHCl] for Ne cyt c.](image2)
Table 3.5 puts the $T_m$ of Ht cyt $c$ and So ScyA with the previously published values for Ht and Pa cyt $c$ (59). It is interesting to note that the melting temperatures scale with the relative growth conditions for each species: Ht is a thermophile, Pa and Ne are mesophiles, and So grows at room temperature. Pathogenic Pa strains are more amenable to the host environment approximately 37 °C.

<table>
<thead>
<tr>
<th>Source Organism</th>
<th>$T_m$ (°C)</th>
<th>$T_m$ Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ht</td>
<td>139</td>
<td>59</td>
</tr>
<tr>
<td>Ne</td>
<td>91</td>
<td>This work</td>
</tr>
<tr>
<td>Pa</td>
<td>80</td>
<td>59</td>
</tr>
<tr>
<td>So</td>
<td>66</td>
<td>This work</td>
</tr>
</tbody>
</table>

Table 3.5 Melting temperature ($T_m$) of Ht, Ne, Pa cyt $c$, and So ScyA.

3.4 Discussion

Our previous work began to shed light on the Met-loss feature exhibited by Ht cyt $c_{552}$ on PGE electrodes. Redox thermodynamics and ligand replacement by the small-molecule imidazole resulted in similar electrochemical responses as those seen for the alkaline form of mitochondrial cyts $c$ (126). In this study we have continued our ligand replacement and redox thermodynamic studies of cyts $c$ homologous to Ht cyt $c_{552}$ in order to discern difference in the factors determining the driving force for electron transfer for both the normal and Met-loss states.

To better understand the electrochemical phenomena we chose to examine two cyt $c$ that are closely related to Ht cyt $c_{552}$: Pa cyt $c_{551}$ and Ne cyt $c_{552}$. Pa and Ht cyts $c$ are mesophile/thermophile pair that has served as a model system for folding, structural
stability, and function in the past \( (56, 57, 59, 64, 65, 124, 129) \). Ht, Pa, and Ne are all members of the cytochrome \( c_8 \) structural family \( (138) \). Among them, \(^1\)H NMR studies have revealed an uncharacteristic compression of heme methyl shifts for Ne and Ht, while Pa is a prototypical member of the cyt \( c_8 \) family \( (103, \ Timkovich, 1994 \#2194, 139) \). The unusual electronic properties have shown that the conformation of the axial Met ligand in Ht and Ne cyt \( c \) is “fluxional.” The \( \delta S \) rapidly switches between the R and S conformations, shown in Figure 3.2 \( (66, 132) \). The variations in Met-donating loops, specifically residue 64 (Pa numbering) impact the local hydrogen bonding system, over all thermal stability \( (65) \), and redox potential \( (129) \). So ScyA was chosen to provide a distantly related bacterial cyt \( c \) within our analysis as it shares between 6-18 % sequence identity to the cyt \( c_8 \) family members in our study (Table 3.2). Moreover, the temperature-dependent broadening of the \(^1\)H NMR hyperfine peaks in addition to the compression of those shifts indicate equilibrium between two species of ScyA on the NMR time scale \( (131) \) in a similar fashion observed for Ht and Ne cyts \( c \) \( (132) \).

### 3.4.1 Ligand binding

Heme-containing proteins occupy a vast range of functional diversity and the additions of exogenous ligands have presented a means to probe the sensitive electronic environment\( (111) \). Addition of imidazole provides a substantially stronger field ligand than the native Met. The Im-Fe adduct is not directly physiologically relevant, however Lys-Fe and His-Fe have been observed as non-native conformations of horse cytochrome \( c \) in the presence of urea and guanidine-HCl, detected by NMR \( (62, 63) \). Redox potentials
for the Im-Fe adducts for all three species in our study are remarkably close to those observed for Ht cy c_{552} (Table 3.3) (126), and the Pa Im-adduct described here develops a potential that agrees with that previously reported (57). Notably, the redox potential of the Im-adduct form becomes more positive as the sequence-identity decreases from that of Ht cy c_{552}. This could possibly reflect the Met-donating loop dynamics and stability post binding. Gray and coworkers demonstrated that as heme solvation increased the redox potential decreased dramatically (50). These findings were recapitulated during studies by Sola; involving N-Acetyl-microperoxidase (AcMP11) (78), a heme protein model system. In that study the Im-AcMP11 adduct experiences a modest decrease in potential versus the water/hydroxo form, a change which may be attributed to the addition of a strong field ligand more than to the overall solvation effects. Larger ligands (like acetyl-methionine or methyl imidazole) begin to decrease the overall solvation of the distal face and result in a large gain in redox potential (78). Inter-loop hydrogen bonding networks are decreased from Ht cyt c_{552} to Pa cyt c_{551} causing the loop to be less rigid (61, 140). No structure is currently available for So ScyA, however the sequence is identical to that of *Shewanella putrefaciens* cytochrome c_5 (Sp cyt c_5) for which the structure is available (141). In this similar protein the change would allow better encapsulation of the Im-Fe causing a substantial increase in the observed redox potential. Our finding are consistent with the mitochondrial alkaline forms (50, 73), cytochrome model systems (78), and our previous work (57, 126).

3.4.2 pH dependence of So ScyA
So ScyA has been found to be pH independent when adsorbed to Au-SAM electrodes. This is in contrast to the other cytochrome c₈ members investigated in Chapters 2 and 3. Ne, Pa, and Ht have distinct pH dependent properties. Ne has a large region of pH independent behavior, while the extremes (< pH 4 and > 9.25) are pH dependent. In an opposite fashion Pa has a pH dependent region approximately neutral pH (7.3 - 6.2), and two independent transitions at the extremes. Ht is unique because it undergoes only one transition, pK_{red} ~pH 4.5, and is otherwise pH independent. This finding reinforces the diversity among closely related proteins.

3.4.3 Redox Thermodynamics and Met-loss Equilibrium

We have examined the redox thermodynamics of three homologous cyts c in order to understand how structural differences affect the fundamental driving force for electron transfer process for both the native and Met-loss species. These findings are summarized in Table 3.4. Overall our findings are in good agreement with those previously published on mitochondrial cyt c and microperoxidases described by Gray (99) and Sola (78, 92, 98). Comparison of the redox thermodynamics of So, Pa, and Ne with Ht (126) shows good correlation with the decrease in structural similarity (Table 3.2). Sola and coworkers showed that enthalpic and entropic terms are largely compensative due to solvation and axial ligation (78). Comparison between the entropic and enthalpic terms show that the driving force between the normal and Met-loss form is largely dictated by the enthalpic term, the redox enthalpy has been largely correlated with the stabilization of the Fe^{2+} state. None of these bacterial cyt c have the break in the $E_{on}$
vs. T (Figure 3.5A) or Gibbs-Helmholtz plots (Figure 3.5B) that has been shown for many mitochondrial species. This could be due to experimental differences largely based on specific technique, solution voltammetry versus PFV where our analyte is an absorbed species \((50, 78)\). As a consequence of examining adsorbed species, we are able to simultaneously measure the populations of the native and Met-loss forms. The populations are directly proportional to the peak height and or the total current passed for each redox couple (peak area). We were able to use these values to calculate an equilibrium constant describing the conversion of the normal form to Met-loss, \(K_{ML}\).

Plotting \(\ln(K_{ML})\) vs \(T^{-1}\) (Figure 3.11)

![Figure 3.11 Plot of \(\ln(K_{eq})\) vs \(T^{-1}\) used to determine the Gibbs free energy of Met loss. The equilibrium constant \((K_{eq})\) was determined by peak intensity of for So cyt \(c_5\) (■), Pa cyt \(c_{553}\) (●), and Ne cyt \(c_{552}\) (▲). Linear regression analysis was used to fit each data set and is shown by the solid line.](image)

yields a slope of \(-\Delta H^\circ/R\) and a \(y\)-intercept of \(\Delta S^\circ/R\). The enthalpy for the conversion is within the regime for subglobal free energies of hydrogen exchange \((\Delta G_{HX})\) and those
determined by direct voltammetry ($\Delta G_{AT}$). Here as in the case of Ht cyt $c_{552}$ (126), the entropic contribution to the overall free energy of conversion is substantial.

### Table 3.6 Comparison of Gibbs free energy for the Met-loss form (ML), sub-global hydrogen exchange of the methionine donating loop (HX), and mitochondrial alkaline form (AT).

<table>
<thead>
<tr>
<th>Species</th>
<th>$\Delta G_{ML,298 \text{K}}$ (kJ mol$^{-1}$)</th>
<th>$\Delta G_{HX}$ (kJ mol$^{-1}$)</th>
<th>$\Delta G_{AT}$ (kJ mol$^{-1}$)</th>
<th>Ref$_{ML}$</th>
<th>Ref$_{HX/AT}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ht cyt $c_{552}$</td>
<td>6.3</td>
<td></td>
<td></td>
<td>Chapter 2, 126</td>
<td></td>
</tr>
<tr>
<td>Ne cyt $c_{552}$</td>
<td>2.7</td>
<td></td>
<td></td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>Pa cyt $c_{551}$</td>
<td>-2.3</td>
<td>26-34</td>
<td></td>
<td>This Work</td>
<td>56</td>
</tr>
<tr>
<td>So ScyA</td>
<td>-6.9</td>
<td></td>
<td></td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td>Hh cyt $c$</td>
<td></td>
<td>26.3</td>
<td></td>
<td></td>
<td>123</td>
</tr>
<tr>
<td>beef heart cyt $c$</td>
<td></td>
<td></td>
<td>52</td>
<td></td>
<td>91</td>
</tr>
<tr>
<td>YCC</td>
<td></td>
<td>48</td>
<td></td>
<td></td>
<td>92</td>
</tr>
</tbody>
</table>

Table 3.6 places this data within the context of free energies associated with other cytochrome c folding units, termed foldons, determined by hydrogen-deuterium exchange ($\Delta G_{HX}$) or for bulk voltammetry ($\Delta G_{AT}$). Regardless, the interaction of bacterial cyts with PGE surfaces appears to lower the free energy requirements to lose the axial methionine ligand.

The thermal melting study has revealed a notable correlation (Table 3.7). Those members of the cyt $c_8$ family that adopt the Met-loss conformation at higher temperatures are also more stable as measured by $T_m$. Furthermore, the conformational plasticity also appears to be correlated with optimal growth temperature. In general, protein thermostability in an organism tends to track with growth temperature (142). Ht
is a thermophile originating from Japanese hot springs (143). So on the far end prefers room temperature growth but will also thrive at normal bacterial culture temperatures (144). Ne is a mesophile. Pseudomonas is a common pathogenic bacterium and thrives at host temperatures (~37 °C) (145).

Table 3.7 The overall stability of cytochrome c folds scale with the Gibbs free energy for the Met-loss transition.

<table>
<thead>
<tr>
<th>Source Organism</th>
<th>Tm (°C)</th>
<th>ΔG_{ML} (kJ Mol^{-1})</th>
<th>Tm Ref</th>
<th>ΔG_{ML} Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ht</td>
<td>139</td>
<td>6.3</td>
<td>26</td>
<td>Chapter 2 and 126,</td>
</tr>
<tr>
<td>Ne</td>
<td>91</td>
<td>2.7</td>
<td>This work</td>
<td>This work</td>
</tr>
<tr>
<td>Pa</td>
<td>80</td>
<td>-2.3</td>
<td>26</td>
<td>This work</td>
</tr>
<tr>
<td>So</td>
<td>66</td>
<td>-6.9</td>
<td>This work</td>
<td>This work</td>
</tr>
</tbody>
</table>

The biological purpose of the Met-loss and alkaline forms are sources of debate. Recently, studies of human cytochrome c demonstrated that mutations at residue 41 decrease the pK\textsubscript{a} for the structural rearrangement to the alkaline conformation. As a consequence the ability to activate Apoptotic protease activating factor 1 (Apaf-1) is enhanced; a key step in the path to apoptosis (146).

3.5 Conclusion

In conclusion, we have shown that the axial ligand of homologous cytochromes c from *Shewanella oneidensis*, *Nitrosomonas europaea*, and *Pseudomonas aeruginosa* are susceptible to replacement by exogenous ligands such as imidazole. The imidazole adducts are similar to the low potential Met-loss feature. The redox thermodynamics for
both the normal and Met-loss forms of So, Ne, and Pa cyt c behave similarly to other species including model systems like microperoxidases. The required driving force for the rearrangement from normal to the Met-loss form is enhanced by the presence of the PGE surface. Ultimately, there is correlation between overall stability, and possibly optimal growth temperature, toward the propensity to adopt the Met-loss form.
Chapter 4 Production and characterization of recombinant

*Nitrosomonas europaea* cytochrome c peroxidase.

4.1 Introduction

The cytochrome c peroxidase from *Nitrosomonas europaea* (Ne CCP) was the first bacterial cytochrome c peroxidase (bCCP) to be isolated in the fully oxidized state with maximal activity. Isolation of the native enzyme by Hooper et al. revealed several unique features with respect to the canonical bCCP enzymes, such as that from *Pseudomonas europaea* (Pa CCP). Foremost, reduction of the high potential heme (H-heme) by one electron, or semi-reduction of the enzyme, cuts activity by two orders of magnitude (41), unlike the Pa CCP, which requires semi-reduction in order to produce any activity. Spectroscopic characterizations by electron absorption and electron paramagnetic resonance (EPR) spectroscopies showed that the H-heme is six-coordinate and low spin, while the low potential heme (L-heme) was five-coordinate and high spin in the fully oxidized state. Activity assays of Ne CCP were additionally not enhanced by the addition of exogenous calcium (Ca\(^{2+}\)), unlike other canonical bCCPs (41). Potentiometric titration also revealed a positive shift in reduction potential of both the H- and L-hemes to +450 mV and -210 mV vs. SHE (41), respectively, as compared to Pa CCP, which displays potentials of +320 mV and -320 mV vs. SHE (38). Finally, crystallographic characterization of the oxidized native enzyme clearly supported the other spectroscopic findings, including the available coordination site at the catalytic L-heme (42). The bCCP from *Methylococcus capsulatus* Bath was isolated in the late
1990s, and was found to be overwhelmingly similar to Ne CCP enzyme, particularly in how it displays full activity in the oxidized form (43).

Comparison of the available bCCP reduction potentials (H- and L-hemes), dependence of activity on redox state, and requirements for calcium to reach maximal activity, delineate two general groups: those that require reductive activation and exogenous calcium, and those that are most active in the fully oxidized state and where a calcium dependence is not observed (Table 4.1).

### Table 4.1 Comparison of bCCPs requirements for semi-reduction, calcium, and redox potential of the high potential and catalytic hemes (41, 43, 147). (*Pseudomonas aeruginosa* (Pa), *Rhodobacter capsulatus* (Rc), *Nitrosomonas europaea* (Ne), and *Methylococcus capsulatus* (Mc))

<table>
<thead>
<tr>
<th>Bacterial Source</th>
<th>Semi-reduction required</th>
<th>Calcium dependence</th>
<th>$E_m$ H-heme (mV)</th>
<th>$E_m$ L-heme (mV)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pa</td>
<td>Yes</td>
<td>Yes</td>
<td>320</td>
<td>-320</td>
<td>44</td>
</tr>
<tr>
<td>Rc</td>
<td>Yes</td>
<td>Yes</td>
<td>270</td>
<td>-310</td>
<td>46</td>
</tr>
<tr>
<td>Ne</td>
<td>No</td>
<td>Not Reported</td>
<td>450</td>
<td>-260</td>
<td>10</td>
</tr>
<tr>
<td>Mc</td>
<td>No</td>
<td>No</td>
<td>432</td>
<td>-250</td>
<td>143</td>
</tr>
</tbody>
</table>

The overall structure and sequence identity between Pa and Ne CCP are significant, both at the level of general protein folds and the orientation and environment of the heme groups. Alignments of the diferric structures highlight a short eleven amino-acid loop adjacent to the L-heme, which has been termed loop 1. Loop 1 provides an axial His ligand in the diferric form that is lost upon reduction of the H-heme (40). Movement to an open conformation provides a coordination site for substrate to bind and enter the catalytic cycle. The corresponding Ne CCP Loop 1 is in the open conformation in the fully oxidized state, as shown in Figure 4.1 (41). Sequence alignment of the loops reveals
several potentially important residues (Figure 4.1C). The Pro residue at the beginning of the loop may add rigidity and promote the closed-loop conformation. There appear to be electrostatic and $\pi$-$\pi$ stacking interactions that may stabilize the closed conformation. Phe92 and Trp61 have aromatic side chains in close proximity and parallel. Also, Trp61 appears to be involved in a hydrogen-bond interaction with the backbone oxygen of Asp94 (Figure 4.2). In Ne and Mc CCP this Asp residue is charge neutral (42, 43). His coordination to the L-heme may also require enhanced flexibility of the Loop 1 provided by the nonconserved Gly residue in Pa CCP, which has a much higher degree of flexibility due to the absence of a side chain.
Figure 4.1 (A) Alignment of the diferric models of Pa CCP (blue, 1EB7) and Ne CCP (red, 1IQC) with the Loop 1 highlighted. (B) The L-heme and Loop 1 zoomed in. (C) Sequence alignment of the Loop 1 for the canonical Pa CCP and the constitutively active Ne CCP. Conserved residues are shown in bold.
Electrochemical investigation by protein film voltammetry (PFV) was previously conducted to investigate the redox chemistry and mechanism of native Ne CCP (44, 45). In the presence of substrate, electrochemical turnover is observed as a sigmoidal wave shape. The catalytic midpoint potential, $E_{\text{cat}}$, was measured to be greater than 500 mV vs. SHE, depending upon the concentration of substrate (45). The catalytic potential is higher than the H-heme reduction potential under non-turnover conditions (+450 mV) (41), and less than that reported by Armstrong for the two-electron generation of Compound I by yeast CCP (+750 mV) (148). The inequality of the three potentials suggests that the measured $E_{\text{cat}}$ is a unique species. The pH dependence of $E_{\text{cat}}$ indicates a complex proton-coupled electron-transfer step associated with the catalytic wave monitored by PFV. The
initial investigation allowed for the proposal of a modified mechanism for Ne CCP that takes into account the high redox potential and full activity in the diferric form (Figure 4.3).

![Proposed catalytic mechanism for Nitrosomonas europaea cytochrome c peroxidase based on Ref (45) (reproduced from Chapter 1, Figure 1.12).](image)

In this chapter we describe a recombinant expression system for the Ne CCP in *E. coli*, its purification, and initial characterization. The recombinant Ne CCP (rNe CCP) will facilitate later work to probe the sequence-structure-function paradigm through mutation of key residues. Our characterization includes electronic absorption spectroscopy, kinetics, EPR, and PFV.
4.2 Materials and Methods

4.2.1 Plasmids and mutagenesis

The *Nitrosomonas europaea* cytochrome c peroxidase (Ne CCP) gene was synthesized by GenScript USA. The synthetic sequence included SacI and HindIII restriction sites for subsequent integration into an expression plasmid, as well as a Tabacco etch virus protease recognition sequence at the N-terminus, and the cytochrome c peroxidase sequence (Figure 4.4). The initial synthetic gene was inserted into the ampicillin resistant (AMP^R^) pUC57 vector (GenScript USA) between exterior EcoRI sites producing the vector pUC57-NeCCP.

The Ne CCP gene was then further sub-cloned into the pMAL-p5x vector (NEB, AMP^R^) between the SacI and HindIII restriction sites, resulting in the construct pMAL-TEV-NeCCP. This construct results in the expression of an N-terminal maltose binding protein.
(MBP) fusion with Ne CCP, however the Factor Xa protease recognition site (native to the pMAL system provided by NEB) has been replaced with the TEV protease recognition sequence -ENLYFQS-. Then, insertion of a GSG linker between the TEV recognition site and the N-terminal sequence of the Ne CCP was required for more efficient TEV protease cleavage. The insertion of the GSG linker was achieved through site-directed mutation, produced by using a QuikChange XL site directed mutagenesis Kit (Stratagene). pMAL-TEV-NeCCP served as the template and the sequences 5’-CTG TAC TTC CAA TCC GGT AGT GGC AAT GAA CCG ATA CAA CC-3’ and 5’-GGT TGT ATC GGT TCA TTG CCA CT A CCG GAT TGG AAG TAC AG-3’ for the forward and reverse primers respectively. The resulting construct was named pMAL-NeCCP-B2.

4.2.2 Expression and purification

The pMAL-NeCCP-B2 plasmid for the production of recombinant Ne CCP was transformed into JM109 E. coli cells bearing the pEC86 plasmid. For optimal expression, a 10 mL pre-culture was grown at 37 °C, 200 rpm, for 12-18 hours. The pre-culture was diluted 1:1000 to inoculate 1-2 L 2xYT cultures and grown for 12-18 hours, 37 °C, 150 rpm. Cells were induced at high OD with 75 mg/L IPTG for 6 hours. All media was supplemented with 100 ug/mL ampicillin and 34 ug/mL chloramphenicol.

Cells were harvested by centrifugation (8000 x g, 4 °C, 15 min), and re-suspended in 20 mM Tris, 200 mM NaCl, 1 mM PMSF, 0.1 mg/mL lysozyme pH 7.4 and disrupted by a single pass through a Microfluidizer™ at 18,000 psi (Microfluidics Model M110P). Cell
debris was removed by centrifugation (10000g, 4 °C, 20 min). The cleared lysate was immediately loaded on to Amylose Resin (New England Bio Labs). Column preparation, MBP-NeCCP fusion binding, and elution was done following the standard protocol. Eluted fractions were pooled and concentrated using Amicon Ultra Centricons (Milipore, 30KDa cutoff). Concentrated protein was loaded onto ÄKTA FPLC equipped with HiPrep™ Sephacryl™ 16/60 S100 column (GE Healthcare). Pure fractions were pooled (as judged by SDS-page gel), concentrated as above, and then clipped using AcTEV protease (Life Technologies). Maltose binding protein was removed by passing the clipping reaction through amylose resin (NEB).

4.2.3 UV-Visible spectroscopy

All optical spectra were collected on a Cary 50 UV-Visible spectrophotometer (Varian). Oxidized samples were prepared by incubation with hexachloroiridate (Sigma). Semi-reduced samples were prepared by the addition of a slight stoichiometric excess of sodium ascorbate.

4.2.4 Determination of iron and heme content

Iron content was determined by atomic absorption spectroscopy using an AA240Z atomic absorption spectrometer (Varian). Calibration standards were prepared by dilution of 1 mg/mL Fe stock solution (Sigma Aldrich) in 0.1% HNO₃. A standard curve was prepared between 0 – 15 ppb Fe. Horse Heart cytochrome c (Sigma Aldrich) was
additionally used as a standard. Experimental samples were diluted in 0.1% HNO₃ and the absorbance was recorded at 248 nm.

Heme content was determined using the pyridine hemochrome assay previously described by Berry (149). Horse heart cytochrome c (Sigma Aldrich) was used as an internal control. Oxidized Ne CCP and Hh cyt c were denatured with sodium hydroxide, treated with pyridine, and oxidized spectra were recorded. Samples were fully reduced by addition of sodium dithionite and the reduced spectra were recorded. The heme content was calculated using the difference between oxidized and reduced spectra at 550 nm using the extinction coefficient ε₅₅₀ = 23.97 mM⁻¹ cm⁻¹ (149).

4.2.5 Activity assays

Enzyme activity assays were performed in 5 mM MES, 5 mM HEPES, 10 mM NaCl, 1 mM CaCl₂ at pH 6. Horse heart cytochrome c (Hh cyt c, Sigma) and Ne cytochrome c₅₅₂ (Ne cyt c) were used as electron donors. Both Hh and Ne cyt c were reduced by treatment with 20 mM sodium ascorbate (Sigma). Excess reductant was removed using a PD-10 desalting column (GE Lifescience). All assays were conducted with fully oxidized enzyme. Horse heart cyt c and Ne cyt c are monitored at 550 nm and 553 nm, respectively, using an extinction coefficient difference between the oxidized and reduced proteins (Δε₅₅₀nm = 21.5 mM⁻¹ cm⁻¹ for Hh and Δε₅₅₃nm = 19.5 mM⁻¹ cm⁻¹ Ne cyt c).
4.2.6 Potentiometric titration

Spectroelectrochemistry was carried out in 5 mM MES 5 mM HEPES 10 mM NaCl 1mM CaCl₂ pH 6. The final protein concentration was 5 µM Ne CCP and the final mediator concentration was 20 µM. Mediator dyes for the potentiometric measurements were ferrocenemethanol( E°' = +440 mV), ferrocene acetic acid (E°' = +375 mV), dichlorophenolindophenol (E°'' = +215 mV), N,N,N',N'-tetramethylphenylenediamine (E°'= +275 mV), Janus green (E°' = -220 mV), Safranine T (E°' = -290 mv), and benzyl viologen (E°' = -350 mV). All mediators were from Sigma Aldrich. Potentials were monitored on a CH Instruments 730b bipotentiostat. The cell was a 1 cm 3 mL quartz cuvette and was used in a three electrode configuration. A resin-body calomel electrode (SCE, Accumet) and platinum wire served as the reference and counter electrodes. A gold rod (Sigma Aldrich) was the working electrode. Spectra were collected on a Cary 50 Bio UV-Visible spectrometer (Varian) equipped with a peltier temperature controller and the cell maintained at 23 °C.

4.2.7 Protein film voltammetry

PFV measurements were carried out on either a PGSTAT12 or PGSTAT30 Autolab (Ecochemie, The Netherlands) fitted with and EDC and FRA modules. A resin-body calomel electrode (SCE, Accumet) and platinum wire were the reference and counter electrodes respectively. Pyrolytic graphite edge-plane (PGE) electrodes served as the
working electrode, and rotation was controlled with an EG&G rotator. The electrochemical cell was maintained at 0 °C by a refrigerated circulator and the reference at room temperature. All potentials were reported with respect to standard hydrogen electrodes (SHE), and were corrected by +242 mV.

PGE electrodes were prepared by mechanical polishing with 1 µm alumina (Buehler), and cleaned by sonication and rinsing with buffer. Typical experiments were done in 5 mM Good’s buffers (MES, HEPES, TAPS, CHES), 100 mM NaCl, and 200 µg/mL polymixin B sulfate. All reagents were from Sigma Aldrich. Polymixin was prepared separately in a 25 mg/mL stock solution and diluted as necessary.

Protein adsorption was achieved by cycling 0.75 to 0.04 V at 10 mV/s and rotating 200 rpm for 20 min in 75 nM rNe CCP in cold buffer. The working electrode was then removed from the protein solution and rinsed with cold buffer to remove excess protein from the electrode surface and body.

4.2.8 EPR Spectroscopy

EPR spectra were collected on a Bruker X-band ElexSYS E 500 spectrometer (9.37 GHz) equipped with an ESR 900 continuous flow liquid helium cryostat. Spectra were recorded at 12K with a modulation frequency of 100 kHz and a 1 G modulation amplitude.
4.3 Results

4.3.1 Molecular Mass

Induction of *E. coli* cells bearing the pMAL-NeCCP-B2 plasmid yielded bright pink pellets after six hours of induction at 37 °C. The Ne CCP-MBP fusion protein eluted from the amylose column in a single band using the recommended procedure from New England BioLabs. After buffer exchange and incubation with TEV protease, the mixture of clipped Ne CCP and MBP was reapplied to an amylose column. Ne CCP dimer was eluted in the flow through while uncleaved and MBP remained bound to the column. Expected product sizes are 38 kDa for the Ne CCP monomer and 80 KDa for the NeCCP-MBP fusion protein. SDS-PAGE gel of the isolated protein migrated as a strong single band at 37 KDa indicating Ne CCP monomer (Figure 4.5).

![Figure 4.5 SDS-PAGE gel of the final fraction collected during the purification of *N. europaea* cytochrome c peroxidase. Stained with Coomassie brilliant blue R-250.](image)
The molecular mass of the non-denatured enzyme was determined by size exclusion chromatography as compared to low molecular weight standards (Sigma). Ne CCP was estimated to be between 75-78 kDa. Upon treatment with 1 mM EGTA the elution was observed later and the size estimated between 40-45 kDa (Figure 4.6).

![Figure 4.6 Elution profile of Ne CCP by size exclusion chromatography on a HiPrep 16/60 Sephacryl S-100 HR.](image)

(Black) Elution profile of standards (Sigma Aldrich) from high to low, aldolase (Mr = 158 kDa), conalbumin (Mr = 75 kDa), ovalbumin (Mr = 43 kDa), carbonic anhydrase (Mr = 29 kDa), and ribonuclease A (Mr = 13.7 kDa). (Red) As purified recombinant Ne CCP elutes with a Mr = 75-78 kDa. (Blue) Recombinant Ne CCP treated with 1 mM EGTA elutes with a Mr = 40-45 kDa. Ne CCP monomer is expected to be 38 kDa.

### 4.3.2 Iron and Heme Composition

Metal analysis carried out on 0.018 and 0.072 μM horse cytochrome c resulted in the observation of 0.25 and 0.093 μM Fe, indicating 1.4 ± 0.2 and 1.2 ± 0.2 Fe/protein. Ne CCP-MBP fusion proteins were similarly analyzed with concentrations of 0.0535 and 0.107 μM. Observed iron was 0.954 and 0.207 μM, indicating 1.8 ± 0.2 and 1.9 ± 0.2 Fe/subunit MBP-CCP fusion. Finally 0.143 μM clipped CCP showed 0.151 μM Fe, indicating a total Fe content of 2.1 Fe/subunit (Figure 4.7). For each concentration,
analyses were conducted in triplicate, with observed errors between 2-12%, based on the standard deviation at each point.

Figure 4.7 Metal analysis carried out by atomic absorption spectroscopy. Iron standards (■), Horse heart cytochrome c standards (•), NeCCP-MBP fusion (▲), and clipped recombinant Ne CCP (▼).

The number of heme prosthetic groups of the bacterial cytochrome c peroxidase from *N. europaea* was determined through the pyridine hemochrome assay described previously (149). The rNeCCP was denatured and treated with pyridine. The total heme content was determined by the absorbance change between the oxidized and reduced states monitored at 550 nm; 2 μM rNe CCP yielded 3.7 μM heme. The resulting heme content is 1.85 ± 0.2 hemes per subunit.
4.3.3 Spectral Properties

The fully oxidized enzyme had absorption maxima at 408 and 550 nm. Upon semi-reduction the Soret maxima became 418 nm and a distinct shoulder appeared at 408 nm. The $\alpha$ and $\beta$ bands arose at 524 and 554 nm respectively (Figure 4.8). Extinction coefficients for Soret and $\alpha/\beta$ bands were found to be similar for those previously reported by Arciero and Hooper (41). The absorbance feature at 640 nm is diagnostic for spin state. In the diferric form the 640 nm feature is not present indicative of low spin. Upon semi-reduction the charge transfer band becomes apparent indicating high spin (41).

The redox behavior of rNeCCP was first studied by spectroelectrochemistry using a homebuilt OTTLE cell and electrochemical mediators. The redox potentials of the two hemes were determined by plotting the log (Ox/Red) species versus potential (Figure 4.9 and Figure 4.10). Consistent with the native enzyme, rNe CCP redox potentials were determined to be $+452 \pm 4$ mV and $-260 \pm 5$ mV.
Figure 4.9 Nernst plot of the optical changes at 554 nm. Mediators were used at 20 µM final concentration; ferrocenemethanol (E°' = +440 mV), ferrocene acetic acid (E°' = +375 mV), dichlorophenolindophenol (E°'' = +215 mV), N,N,N',N'-tetramethylphenylenediamine (E°''' = +275 mV). The titration was done in both the oxidative and reductive directions.

Figure 4.10 Nernst plot of the optical changes at 554 nm. Mediators were used at 20 µM final concentration; Janus green (E°'' = -220 mV), safranine T (E°''' = -290 mV), and benzyl viologen (E°'''' = -350 mV). The titration was done in reductively.
Figure 4.11 rNeCCP Wt displays the spin-state transition from low to high spin upon semi-reduction. This transition may be monitored at 640 nm. Shown here are the oxidized spectra (Bottom) and semi-reduced species (Top).

4.3.4 Electron Paramagnetic Resonance

Electron paramagnetic resonance (EPR) of the oxidized (Figure 4.12) and semi-reduced (Figure 4.13) enzyme are similar to those of the native enzyme and other bacterial cytochrome c peroxidases. The oxidized spectra indicate the presence of a one high spin and one low spin species. The high spin signals appear at $g = 6.2, 5.7, \text{ and } 2$. The remaining signals are typical of low spin species at $g = 2.8, 2.3, 1.5$. The signal at $g = 3.4$ is indicative of a highly axial (or anisotropic) low spin species (HALS). The MBP-rNeCCP fusion appears to have a significantly enhanced HALS signal. Upon semi-reduction the high spin and HALS signals are significantly reduced. The low spin signals are unchanged.
Figure 4.12 EPR Spectra of the oxidized native and recombinant Ne CCP. Native Ne CCP from Arciero and Hooper (A) reproduced without permission (41), clipped rNeCCP (B) and rNeCCP-MBP fusion (C). The native protein was 400µM. Both recombinant samples were 250µM. The signal at g = 4.3 results from junk iron within the sample.
Figure 4.13 EPR Spectra of the semi reduced native and recombinant Ne CCP. Native Ne CCP from Arciero and Hooper (A) reproduced without permission (41), Clipped rNeCCP (B) and rNeCCP-MBP fusion (C). The native protein was 250µM. Both recombinant samples were 250µM. The signal at g = 4.3 results from junk iron within the sample.
4.3.5 Protein Film Voltammetry

Direct electrochemical analysis of rNe CCP yielded a highly reversible catalytic response in the presence of hydrogen peroxide (Figure 4.14). The stable films give reversible sigmoidal waves centered at the catalytic redox potential, \( E_{\text{cat}} \). We have measured \( E_{\text{cat}} = 510 \text{ mV} \) or greater depending on peroxide concentration at pH 6.5.

\[ \text{Current (\mu A)} \]

![Figure 4.14 Catalytic voltammetry of rNeCCP with increasing amounts of hydrogen peroxide at pH 6.5, } \nu = 20 \text{ mV/s, } T= 0 \text{ °C, and the electrode rotating at 1800 rpm.}

The magnitude of the limiting current (\( i_{\text{lim}} \)) may be treated as an enzymatic velocity and saturates in accord with typical Michaelis-Menten kinetics. The \( K_m \) is in agreement with the native enzyme and other reported diheme peroxidases (45, 150, 151); here the electrochemically determined \( K_m \) value was 57 ± 2 \( \mu \text{M} \) for hydrogen peroxide. Typically, the catalytic velocity (\( k_{\text{cat}} \)) would be calculated with respect to the electroactive surface coverage. However, under non-turnover conditions there is an absence of such features, as in the prior case of the native enzyme; the presence of such “non-turnover features”
would indicate the number of enzyme molecules participating in adsorbed electrochemistry. Given the absence of non-turnover features, we have assumed that the coverage is less than 0.5 pmol/cm$^2$, and calculated that $k_{\text{cat}}$ is over 1000 s$^{-1}$ in agreement with the reported native enzyme (45).

The pH-dependent behavior mirrors that of the native enzyme as well (Figure 4.15). The redox potential is dependent on pH and fits well to a two pK$_a$ model (Eq 4.1). This model yields two pK$_a$ values of 6.7 ± 0.3 and 8.5 ± 0.2 for the reduced and oxidized species.

![Figure 4.15 pH dependence of $E_{\text{cat}}$ determined for the rNeCCP (▼) and the native Ne CCP (●) in the presence of 100 μM. Data for the native enzyme is from Bradley et al. (45). Data was fit to equation 4.1 which represents the binding of two protons through the course of a one-electron process (n = 1).](image)
\[ E = E' + \frac{2.303RT}{nF} \log \left( \frac{[H^+]^2+[H^+]K_{red}}{[H^+]+K_{ox}} \right) \quad \text{Eq 4.1} \]

4.3.6 Steady state catalytic properties

Steady state solution kinetics was determined for the recombinant fusion protein and the clipped rNeCCP using the monoheme cytochromes \( c \) from horse heart (Hh cyt \( c \), Figure 4.16) and \( Ne \) as electron donors. The rNeCCP was active in both the oxidized and reduced states, and the addition of exogenous calcium did not enhance the activity. All of the kinetics reported here used the oxidized enzyme and are summarized in Table 4.2.

![Figure 4.16 Michaelis-Menten plots of the rNe CCP clipped (■) and MBP fusion protein (■) with Horse heart cytochrome \( c \) as the electron donor (A). The rNe CCP clipped (●) and MBP fusion protein (●) with Nitrosomonas europaea cytochrome \( c \) as the electron donor (B).](image)
<table>
<thead>
<tr>
<th>Electron Donor</th>
<th>Parameter</th>
<th>rNe WT</th>
<th>rNe WT-MBP</th>
<th>nNe WTa</th>
</tr>
</thead>
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<tr>
<td>Hh cyt c</td>
<td>$K_m$ (μM)</td>
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<td>0.003</td>
<td>N.R.</td>
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<td>$k_{cat}^{obs}$ (s$^{-1}$)</td>
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<td>0.331</td>
<td>3</td>
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<td></td>
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<td>0.004</td>
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<td>$k_{cat}^{obs}$ (s$^{-1}$)</td>
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<td>0.347</td>
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<td></td>
<td>$k_{cat}/K_m$ (x10$^6$ M$^{-1}$ s$^{-1}$)</td>
<td>352</td>
<td>87</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2 Kinetic parameters of the rNeCCP and native enzyme with different electron donors. aFrom Ref (41).

4.4 Discussion

Two general classes of bacterial diheme peroxidases are have been described within the literature. *Pseudomonas*-like enzymes require reductive activation and are dependent on the presence of calcium for maximal activity (147, 151-154). *Nitrosomonas*-like enzymes are constitutively active (41, 43). Very little has been published with respect to the native *Nitrosomonas europaea* and *Methylococcus capsulatus Bath*, because (in part) the native enzyme purification is labor intensive and a significant amount of protein is required for some spectroscopic techniques such as stopped-flow. In this Chapter we have described the characterization of recombinant *Nitrosomonas europaea* diheme cytochrome c peroxidase. The recombinant product purifies as homodimer and has a subunit mass of approximately 38 kDa. Each subunit contains two iron and two c-type hemes determined by atomic absorption and the
pyridine hemochrome assay. Combined spectroelectrochemical analysis (Figure 4.9 and Figure 4.10) and EPR spectroscopy indicate that one heme is high potential \( E_m = +452 \) mV) and low spin while the other is low potential \( E_m = -260 \) mV) and high spin in the fully oxidized state, while the EPR spectra are highly similar to those reported by Arciero and Hooper (41). *Pseudomonas*-like bCCPs undergo a change in spin state in addition to the conformational change upon semi-reduction. The diagnostic UV-visible feature at 640 nm is absent in the fully oxidized form, but clearly apparent in the semi reduced state.

The catalytic properties of the rNe CCP are similar to the reported properties of native enzyme by both protein film voltammetry (45) and in the initial solution measurements (41). However there is clear discrepancy between values determined by the separate methods. In the steady-state regime we were able to compare the kinetics between several electron donors; Hh cyt c and a mono heme cyt c from *N. europaea*. The native redox partner for Ne CCP is not proven at this time although several mono-heme cytochromes are present in the *N. europaea* genome. Hh cyt c is a common electron donor that has been unitized with other bCCPs (155). The Ne cyt c\textsubscript{552} utilized in this assay is highly abundant in the native organism, and has been extensively studied as a foil for protein folding and for understanding the driving-force electron-transfer chemistry. The redox potential for these donors (129) are close and when compared in activity assays result in very similar kinetic parameters. The MBP-fusion protein catalytic efficiency \( k_{cat}/K_m \) is severely hampered by the presence of the maltose binding protein;
affinity for peroxide is relatively unchanged. In the absence of MBP we were able to observe turnover equal to that of the native enzyme. The initial report did not include a $K_m$ value for hydrogen peroxide, here we observed a very tight affinity for peroxide of approximately 10 nM. At high concentrations of peroxide ($H_2O_2 < 100 \mu M$) bubbling was evident in the reaction cuvette suggesting the possibility of catalase activity; this would suggest that Ne CCP may be a bifunctional catalase-peroxidase.

4.5 Conclusion

In Chapter 4 we have shown that the diheme peroxidase from *N. europaea* can be produced in a novel recombinant system, and is catalytically competent compared to the native enzyme. These efforts will allow us to probe more deeply into the catalytic mechanism proposed through PFV studies. In Chapters 5 and 6 we will use the rNe CCP as a platform for site directed mutagenesis and understanding ubiquitously conserved Trp residues and investigation of the strongly proton-coupled electron transfer observed by PFV.
Chapter 5 A kinetic and electrochemical investigation into the roles of conserved tryptophan, glutamine, and glutamic acid residues in *Nitrosomonas europaea* cytochrome c peroxidase

5.1 Introduction

In Chapter 4 we established that our recombinant *Nitrosomonas europaea* cytochrome c peroxidase (rNe CCP) and the native enzyme (nNe CCP) have the same biophysical, kinetic, and electrochemical characteristics. In Chapter 5 we use site-directed mutagenesis to elucidate the function of several highly-conserved residues among bCCPs. Two constitutively conserved Trp residues are thought to play key functional roles. For example, as shown in Figure 5.1 the most likely pathway of electron transfer between the two hemes of all bCCP enzymes is through Trp82.

![Figure 5.1 Monomers of Ne CCP (PDB 1IQC). Trp 82 (blue) is situated between the H- and L-hemes and the proposed route of electron transfer. Calcium ion shown in yellow.](image-url)
Trp61 is also strictly conserved, though found on Loop 1; it is less evident how the position can be assigned to a specific function (Figure 5.2A). The high redox potential of the native and recombinant catalytic PFV, in addition to maximal solution activity when fully oxidized continues to support the possibility that Trp61 play a role in the generation of tryptophanyl cation radical, as in yeast CCP, or be part of the stabilization of the ‘open’ conformer of Loop 1 (Figure 5.2). In order to probe function of these two Trp residues, modest systematic mutations have been made (Tyr, Phe, Ala) in order minimize overall structural perturbation. Functional impacts can then be assessed through steady-state kinetic studies using Hh and Ne cyts c as electron donors.

Within the L-heme pocket two residues, Glu92 and Gln102 side-chains are directed toward the heme center and the amide/carboxylate groups come within five Å of iron (Figure 5.2B). These residues are highly conserved among bCCPs and could serve as
analogous residues to His/Arg pair found in monoheme peroxidases. Within monoheme peroxidases the His residue serves as a general acid/base to facilitate proton transfer, and the Arg serves to balance the partial negative charge during the compound 0 transition state. Previous studies in *R. capsulatus* have shown that removal of these residues results in the complete loss of activity. In our study we have made more modest mutations, removing a methylene from each side chain. We have analyzed three mutations by both solution kinetics and PFV: Q92N, E102D, and the Q92N/E102D double mutant.

5.2 Materials and Methods

5.2.1 Mutagenesis and purification of Ne CCP mutants

The mutations were prepared using the QuiChange XL site directed mutagenesis Kit (Stratagene). pMAL- NeCCP B2 served as the template and the sequences for the forward primers are in Table 5.1. The resulting constructs were named pNeCCPB2-X##Y (ex. pNeCCPB2-W61Y).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Forward Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>W61Y</td>
<td>5’-CCACCTCGATCGGGCACAAATATTCAGCAAGGCCCGATCAATG-3’</td>
</tr>
<tr>
<td>W61F</td>
<td>5’-CCACCTCGATCGGGCACAAATTTTCAGCAAGGCCCGATCAATG-3’</td>
</tr>
<tr>
<td>W61A</td>
<td>5’-CCACCTCGATCGGGCACAAAGCGCAGCAAGGCCCGATCAATG-3’</td>
</tr>
<tr>
<td>W82Y</td>
<td>5’-GCATGAATCTGGCAGCAATTTCTATGATGGCAGAGCC-3’</td>
</tr>
<tr>
<td>W82A</td>
<td>5’-GCATGAATCTGGCAGCAATTTCTGCAGAGGCCAGAGCC</td>
</tr>
<tr>
<td>Q92N</td>
<td>5’-GCCAAAGACCTGAAAGAAAACGCTGCCGGCCCTTGC-3’</td>
</tr>
<tr>
<td>E102D</td>
<td>5’-CGATTGCAACCCGAAGGATATGGCCTCTACCCATG-3’</td>
</tr>
</tbody>
</table>

Table 5.1 Forward primers used for the production of site directed mutations at Trp 61, Trp 82, Q92, and E102.
5.2.2 Activity assays

Peroxidase activity assays were conducted as previously described in Chapter 4.2.6 unless otherwise noted. The assays for the mutations W61F, W82Y, and Q92N/E102D required 140 nM Ne CCP and 40 µM electron donor.

5.2.3 Electrochemical Methods

All PFV measurements were carried out on a PGSTAT30 (Ecochemie) electrochemical analyzer equipped with FRA and ECD modules. A glass cell with an integrated water jacket was used in a three-electrode configuration. Cell temperature was maintained by a refrigerating circulator. A resin-body calomel electrode (Accumet) and platinum wire were used as the reference and counter electrodes, respectively. The calomel reference was maintained at a constant temperature (293 ± 0.5 K). Potentials are reported vs. standard hydrogen electrode (SHE) and corrected by +242 mV. Typical experimental conditions and adsorption of Ne CCP mutations are described in Chapter 4.2.7.

5.2.4 Potentiometric titrations

Spectroelectrochemistry was carried out in 5 mM MES, 5 mM HEPES, 10 mM NaCl, and 1 mM CaCl₂ pH 6. The final protein concentration was 5 µM Ne CCP and the final mediator concentration was 20 µM. Mediator dyes for the potentiometric measurements were ferrocenemethanol (E°’= +440 mV), ferrocene acetic acid (E°’ = +375 mV), dichlorophenolindophenol (E°’ = +215 mV), N,N,N',N’-
tetramethylphenylenediamine ($E^{o'}= +275 \text{ mV}$), Janus green ($E^{o'}= -220 \text{ mV}$), Safranine T ($E^{o'}= -290 \text{ mv}$), and benzyl viologen ($E^{o'}= -350 \text{ mV}$). All mediators were from Sigma Aldrich. Potentials were monitored on a Denver Instruments Model 215 meter equipped with an Ag/AgCl electrode. Potentials were adjusted $+200 \text{ mV}$ versus standard hydrogen electrode (vs SHE). The cell was a 1 cm 3 mL quartz cuvette used in a three electrode configuration. Spectra were collected on a Cary 50 Bio UV-Visible spectrometer (Varian) equipped with a Peltier temperature controller and the cell maintained at 23 °C.

5.3 Results

5.3.1 Protein expression

Expression of the mutant constructs proceeded with similar magnitude as the native construct (see prior Chapter). The exception is the mutation Trp82Phe. Despite a plethora of expression conditions and cell lines, this mutation was not tolerated and was unable to be expressed or purified.

During the protein purification process we looked to see if the mutations retained the change in spin-state upon semi-reduction. For each mutation we were not able to observe the characteristic band at 640 nm for the diferric form. However in the semi reduced state there is a clearly visible transition at 640 nm exemplified in Figure 5.3 for the L-heme pocket mutations. These findings are in Table 5.2.
Figure 5.3 rNeCCP displays the spin-state transition from low to high spin upon semi-reduction. This transition may be monitored at 640 nm. Shown here are the oxidized spectra (Bottom) and semi-reduced species (Top) clearly showing the feature at 640 nm. Spectra shown here are for Q92N (A) E102D (B) and Q92N E102D (C).

Table 5.2 rNeCCP and all of the variants investigated in this chapter undergo the spin-state transition from low to high spin upon semi-reduction.

<table>
<thead>
<tr>
<th>rNeCCP</th>
<th>Peak at 640 nm</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Oxidized</td>
</tr>
<tr>
<td>WT</td>
<td>No</td>
</tr>
<tr>
<td>W61A</td>
<td>No</td>
</tr>
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<td>No</td>
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</tr>
<tr>
<td>E102D</td>
<td>No</td>
</tr>
<tr>
<td>Q92N E102D</td>
<td>No</td>
</tr>
</tbody>
</table>
5.3.2 Steady state kinetics

Trp 61 and Trp 82 are strictly conserved among bCCPs. Elimination of the aromatic side chain through mutation to Ala (W82A) completely abolishes activity in the standard solution assay (Chapter 4.3.6 and 5.3.2). Mutation to Tyr reduces the activity 1000 fold.

Similarly, replacement of the Trp 61 aromatic side chain with a methyl group abolishes activity. Retaining the aromaticity through mutation to Tyr and Phe results in an increase in $K_m$ from low nanomolar to micromolar. These results are shown in Figure 5.4 and the kinetic parameters summarized in Table 5.3.

![Figure 5.4 Michaelis-Menten plots of the rNe CCP W61Y (■), W61F (■) and W82Y (■) with Horse heart cytochrome c as the electron donor (A). rNe CCP W61Y (●), W61F (●) and W82Y (●) with Nitrosomonas europaea cytochrome c as the electron donor (B).](image)

The side-chain carboxylate/amide goups of Gln92 and Glu102 point directly toward the iron of the peroxidatic heme (Figure 5.2). The Michaelis-Menten plots are
shown in Figure 5.5 and summarized in Table 5.3. We have made modest mutations that remove a methylene group from each residue. Gln92Asn is a polar uncharged mutation that gives a modest order of magnitude loss in activity. The $K_m$ for peroxide is also raised significantly from 0.008 μM to 36 μM.

Glu102Asp mutation results in a reduction of $k_{cat}$ by half, however the impact on $K_m$ is much more pronounced (~2 μM). The double mutation, Q92N/E102D, did not yield a additive change, however the overall changes in both $K_m$ and $k_{cat}$ were the largest of this series of mutations. These mutations effecting catalysis did not show drastic changes in the overall pH dependence. In general the maximal activity was observed between pH 6-6.5 with activity falling off substantially at pH < 6 or pH > 7.5. The pH-dependent steady state kinetics are summarized in Figure 5.6.
Figure 5.6. pH dependence of the relative activity of rNe CCP WT (▲), Q92N (■), E102D (●), and Q92N E102D (■) with horse heart cytochrome c as the electron donor. Activity was normalized using the maximal velocity for each species as 100% and comparison of activity to that value.
<table>
<thead>
<tr>
<th>Electron Donor</th>
<th>Parameter</th>
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<th>rNe WT-MBP</th>
<th>nNe WT&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>W61F</th>
<th>W61A</th>
<th>W82Y</th>
<th>W82A</th>
<th>Q92N</th>
<th>E102D</th>
<th>Q92N E102D</th>
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</thead>
<tbody>
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<td>Hh cyt c</td>
<td>K&lt;sub&gt;m&lt;/sub&gt; (μM)</td>
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<td>0.003</td>
<td>N.R.</td>
<td>2.2</td>
<td>9</td>
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<td>0.008</td>
<td>0.0011</td>
<td>-</td>
<td>0.001</td>
<td>-</td>
<td>0.9</td>
<td>1.69</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt; (x10&lt;sup&gt;6&lt;/sup&gt; M&lt;sup&gt;-1&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>352</td>
<td>87</td>
<td>0.0030</td>
<td>0.00015</td>
<td>-</td>
<td>0.14</td>
<td>-</td>
<td>0.027</td>
<td>0.78</td>
<td>0.00051</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3 Steady state kinetic parameters for wild type rNe CCP (Chapter 4), Trp, and proton coupled electron transfer mutations. N.R. = Not Reported.
5.3.3 Electrochemical Methods

5.3.3.1 Protein film voltammetry

The protein variants bearing mutations within the heme pocket were adsorbed to PGE electrodes as previously described. Peroxide up to 1 mM was added to the cell solution but we were unable to observe the sigmoidal wave typical of bCCP electrochemistry (Figure 4.14). However, a single non-turnover signal was observed for each variant, largely at the identical potential. At pH 7 the redox potential was measured to be +270 ± 3 mV (Figure 5.7). The pH-dependent voltammetry resulted in a linear dependence on pH over pH 3.5 to 10.5, and slope of -58 mV/pH unit. At the pH extremes enzyme adsorption became less stable. Critically, this data does not allow for meaningful comparison with the rNe CCP wild type. It demonstrates the sensitivity to environment. The rNe CCP did not display any non-turn over signals. In the absence of substrate, rNe CCP mutations Q92N and E102D had observable the non-turnover signals when adsorbed on PGE electrodes. No activity was observed in the presence of peroxide.

![Graph showing pH dependence of redox potential](image)

Figure 5.7 pH dependence of redox potential. Slope = -58 mV/pH unit. rNe CCP Q92N (●), E102D (▲), and Q92N E102D (■). Voltammograms were collected at 0 °C, pH 6.5, 5mM MES, HEPES, TAPS, CHES, 100mM NaCl, 1 mM Ca²⁺, v = 100 mV/s.
5.3.3.2 Potentiometric titrations

In response to the puzzling PFV data we extended our electrochemical investigation to include potentiometric titration. We monitored the change in absorbance and 553 nm as described in Chapter 4. A Summary of these findings is found in Table 5.4. In general, we observed a depression of the reduction potential for both hemes. The double mutation variant shows nearly additive changes in potential, which is striking based on the overall non-additive steady-state kinetics. For the low potential heme the steady decrease in redox potential is consistent with an increase in heme pocket solvation.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Potential (mV vs SHE)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-Heme</td>
<td>L-Heme</td>
</tr>
<tr>
<td>WT</td>
<td>+452 ± 4</td>
<td>-260 ± 5</td>
</tr>
<tr>
<td>Q92N</td>
<td>+438 ± 6</td>
<td>-263 ± 3</td>
</tr>
<tr>
<td>E102D</td>
<td>+445 ± 5</td>
<td>-272 ± 5</td>
</tr>
<tr>
<td>Q92N E102D</td>
<td>+432 ± 4</td>
<td>-277 ± 5</td>
</tr>
<tr>
<td>W61A</td>
<td>+442 ± 4</td>
<td>-272 ± 5</td>
</tr>
<tr>
<td>W61F</td>
<td>+448 ± 4</td>
<td>-262 ± 6</td>
</tr>
<tr>
<td>W61Y</td>
<td>+438 ± 6</td>
<td>-268 ± 5</td>
</tr>
<tr>
<td>W82Y</td>
<td>+421 ± 4</td>
<td>-273 ± 3</td>
</tr>
<tr>
<td>W82A</td>
<td>+411 ± 7</td>
<td>-277 ± 5</td>
</tr>
</tbody>
</table>

Table 5.4 Redox potentials for the H- and L-hemes determined by potentiometric titration. Mediators were used at 20 µM final concentration: ferrocenemethanol (E°' = +440 mV), ferrocene acetic acid (E°' = +375 mV), dichlorophenolindophenol (E°'' = +215 mV), N,N,N',N'-tetramethylphenylenediamine (E°' = +275 mV), Janus green (E°' = -220 mV), Safranine T (E°' = -290 mV), and benzyl viologen (E°' = -350 mV). The titrations were done under reductive conditions using sodium dithionite.

5.4 Discussion

In Chapter 5 we assessed the role of several constitutively conserved residues through mutation and steady-state kinetic analysis. Among all bacterial cytochrome c peroxidases (bCCPs) there is a conserved Trp residue situated between hemes; Trp 82 in
Ne CCP (Figure 5.1). We hypothesize that this Trp is the likely conduit of electron transfer between the H- and L-hemes. Gray and coworkers described the distance limitations for direct electron transfer between species. Beyond approximately 15 Å the likelihood for through-space electron transfer is very low. The distance between iron centers in Ne CCP is too great for direct electron transfer, while the edge to edge distances from H-heme to Trp to L-heme are well within the accepted distance for direct electron transfer (Figure 5.8). Alternatives would involve a through protein route, but after inspection of the structural model no obvious route exists. Mutation of Trp82 will allow us to assess how electrons are shuttled from the H- to L-heme.

**Figure 5.8** Cartoon representation of the H- and L-hemes of Ne CCP with Trp 82 shown in blue. Interdomain calcium is shown in yellow. Model and distances were measured using PyMol and PDB 1IQC.

*Nitrosomonas*-like peroxidases are active in the fully oxidized form. The putative catalytic mechanism hypothesizes production of a cation radical species upon the initial interaction with substrate (45) similar to the intermediates for P450 and monoheme peroxidases. The nature of this intermediate is not obvious. Our remaining mutations
have been made to help elucidate the nature of the radical species and residues that help facilitate the generation of compound I. The formation of compound I in HRP results in a stable porphyrin cation radical, which has characteristic spectroscopic features including a bleaching of the soret and an increase in absorbance between 600-650 nm (156). In the case of Ne CCP we hypothesize that upon binding of peroxide a compound I-like intermediate is formed along with a porphyrin cation or Trp radical. Unfortunately, the presence of the second heme makes the direct comparison with other optical spectra difficult.

Compound I is comprised of a Fe(IV)-oxo species and cation radical housed either on the porphyrin or an amino acid. Trp 61 is conserved within the middle of loop 1 and a good candidate for housing a radical. Loop 1 undergoes a series of conformational changes during reductive activation in *Pseudomonas*-like bCCPs. In constitutively active bCCPs, loop 1 may also be dynamic, but not inhibit substrate binding.
Monoheme peroxidases have a conserved His/Arg pair that facilitates generation of compound I (Figure 5.9). The His residue functions as a general acid/base to shuttle a proton and the Arg stabilizes charge within the pocket before water is liberated. Two residues within the catalytic heme pocket appear to be poised in a similar fashion. Q92 and E102 are within 5 Å of the L-heme iron. In Pa CCP these residues are further away from iron in the diferric state but are brought to a similar range upon semi-reduction. Previous attempts to study these residues have met with difficulty. Mutations within the heme pocket of the bCCP form R. capsulatus resulted in no measurable activity (147). We chose to make two conservative mutations to investigate the function of these residues. Removal of a methylene from each side chain was chosen to minimize changes in the local environment, and resulted in the design of the mutations Q92N and E102D.
5.4.1 Electron transfer pathways

The mutation series Trp to Tyr, Phe, or Ala was used probe the electron transfer between H- and L-hemes. We were able to successfully express all mutations except Trp82Phe. Curiously, So CCP would not tolerate this mutation either (157). Trp82Tyr significantly slowed the peroxidase activity while not affecting the \( K_m \), suggesting that we have slowed transfer between the H- and L-hemes while not adversely affecting peroxide binding. Trp82Ala completely removes measureable steady-state peroxidase activity. These two results suggest that Trp 82 acts as the conduit between the H- and L- hemes and other pathways do not exist or are too slow for measureable activity.

5.4.2 Role of Trp 61 within loop 1

![Figure 5.10 Model of the Ne CCP dimer (PDB 1IQC) highlighting the proximity of neighboring loop 1 Trp residues. Hemes and Trp 61s are shown in orange.](image)
Trp61 is the most likely candidate for an amino-acid based radical similar to that observed in yeast peroxidase cytochrome c peroxidase (yCCP). If this residue is essential for catalysis mutation should abolish activity. Similar to Trp82, we made the mutation series: Trp to Tyr, Phe, or Ala. For W61Y and W61F we observed a three order of magnitude reduction in catalytic velocity and a similar increase in $K_m$. W61A resulted in no measurable activity. It appears that W61 has two potential functions. First, it appears to function as a partial regulator for substrate affinity. W61Y is approximately three times as active as W61F. Tyr and Phe are very similar in structure, but the Tyr has a hydroxyl substituent which could provide direction through electrostatics in a closed conformation. It could also function as part of the proton donor network. Alternatively, Trp 61 may also participate in subunit cross-talk. The model produced from the diferric form (Figure 5.10) clearly shows Trp 61 side chain oriented toward the neighboring H-heme of the dimer. Regardless of these possibilities we did see a drastic loss of activity. This suggests that the residue is not critical for generating a radical species during catalysis.

5.4.3 Formation of compound I

Close examination of the Pa and Ne active sites reveal two residues that are poised to facilitate the formation of compound I. Examination of the Ne model reveals that Glu 92 and Gln 102 are within 5 Å of the heme iron. In Pa these residues are approximately 15 Å apart when fully oxidized, and are brought closer during semi-reduction. Steady-state kinetic measurements revealed striking differences in $K_m$ and $k_{cat}$. 
Gln92Asn conferred a large increase in $K_m$ 35.6 vs. 0.008 μM for the wild type, but only a 10-fold decrease in $k_{cat}$. This mutation is of a polar but uncharged side chain, and the removal of the methylene group could give the substrate hydrogen peroxide more room to enter/exit the active site and find the proper orientation for catalysis. The mutation Glu102Asp confers a pK$_a$ change from 4.15 to 3.71 of the free amino acid residues (158) and while there is a modest increase in $K_m$ (1.82 μM), we only observed reduction in $k_{cat}$ by half. The $K_m$ increase might be due to the increase in the volume of the binding pocket for the substrate, i.e. non-productive binding. The impact of the active site volume on proton transfer appears to be less overall than that of the Gln92Asn mutation which is proposed to provide orientation. The double mutation does not have a strictly additive effect but the 187 μM $K_m$ was surprising. It is important to note that mutations to analogous residues in Rc CCP completely abolish activity (147).

It was our hope to investigate these mutations through catalytic PFV studies, but these variants showed no activity in the presence of hydrogen peroxide up to 1 mM. Notably, all three mutations yielded reversible non-turnover signals when adsorbed onto PGE surfaces. In most cases only the low potential peaks were present. All three species (Gln92Asn, Glu102Asp, and double mutation) exhibit proton-coupled electron transfer typical of a 1e⁻:1H⁺ with slope -58 mV/pH unit which is very close to the ideal -59 mV/pH. The observed redox potentials using PFV do not conform to the measured potential via spectroelectrochemistry of the rNe CCP nor do they correspond to potentials similar to the catalytic potential ($E_{cat}$). The potential is close to that reported for the H-
heme of *R. capsulatus* (159), however a correlation is unclear. This may be due to disruptive interactions with the PGE surface. In Chapters 2 and 3 we have shown that at times the PGE surface is non-innocent and can cause local structural changes (126).

5.4.4 Kinetic models and assumptions

In general, the kinetic data for bCCP catalysis does not fit the traditional Michaelis-Menten model of enzyme catalysis. An unspoken assumption that we have made in these experiments (present and past) is that upon interaction with peroxide CCP immediately reacts to form products irreversibly, and there is no substrate-enzyme dissociation. From the perspective of this study that fact complicates the kinetic analysis in several ways. Primarily, the parameters $K_m$ and $k_{cat}$ may not be reporting the actual values with respect to the typical assumptions and assay conditions. Furthermore this circumstance may account for the discrepancy between kinetic parameters determined through PFV studies (Chapter 4).

Enzyme kinetics are classically presented as a two-step model; the equilibrium or steady-state models. Both models are reducible to the same expression presented by Michaelis and Menten in 1913 (160). With each case they have similar, yet different assumptions. In general, the classic enzymatic reaction may be modeled as a reversible binding of substrate (S) to enzyme (E), and then upon favorable binding the transformation to product (P) (eq 5.1).

$$E + S \xleftrightarrow{k_s} ES \xrightarrow{k_{cat}} E + P$$  

eq 5.1.
The speed at which an enzyme can turnover is described by the first order rate constant constant $k_{cat}$ (or turnover number). The rate of catalysis ($v$) can then be expressed as the concentration of enzyme-substrate complex, given by the equilibrium constant $K_s$ (eq 5.2) and turnover $k_{cat}$ to yield eq 5.3.

$$\frac{[E][S]}{[ES]} = K_s \quad \text{(eq 5.2)}$$

$$v = k_{cat}[ES] \quad \text{(eq 5.3)}$$

The total free enzyme may be related to the total enzyme concentration and that bound in the enzyme-substrate complex by eq 4.

$$[E] = [E]_0 - [ES] \quad \text{(eq 5.4)}$$

By substitution

$$[ES] = \frac{[E]_0[S]}{K_s+[S]} \quad \text{(eq 5.5)}$$

And

$$v = \frac{[E]_0[S]k_{cat}}{K_s+[S]} \quad \text{(eq 5.6)}$$

Michaelis and Menten made several assumptions in deriving this relationship:

1. The binding step is fast which establishes an equilibrium state rapidly. Further the catalytic step is slower than the binding step.
2. The substrate concentration must be sufficiently large such that is remains essentially constant. If $[S] \gg [E]_0$ then $[ES]$ is negligible.
3. The back reaction of converting product back to substrate ($P \rightarrow S$) is negligible.

In the simplest case, the Michaelis-Menten constant ($K_m$) is analogous to $K_s$. If the catalytic step is not much slower than the equilibrium of substrate, enzyme, and ES
complex then the relationship $K_m = K_s$ is no longer valid. The model could be expressed as rate constants for substrate binding as shown in eq 5.7.

$$E + S \overset{k_1/k_{-1}}{\longrightarrow} ES \overset{k_2}{\rightarrow} E + P$$  \hspace{1cm} (eq 5.7)

If we then assume again that the ES complex is constant over time

$$\frac{d[ES]}{dt} = 0 = k_1[E][S] - k_2[ES] - k_{-1}[ES]$$  \hspace{1cm} (eq 5.8)

And substitution of eq 5.4.

$$[ES] = \frac{[E]_0[S]}{[S] + (k_2 + k_{-1})/k_1}$$  \hspace{1cm} (eq 5.9)

And

$$v = k_2[ES]$$  \hspace{1cm} (eq 5.10)

Finally yield

$$v = \frac{[E]_0[S]k_2}{[S] + (k_2 + k_{-1})/k_1}$$  \hspace{1cm} (eq 5.11)

Where

$$K_m = \frac{k_2 + k_{-1}}{k_1}$$  \hspace{1cm} (eq 5.12)

And

$$K_m = K_s + \frac{k_2}{k_1}$$  \hspace{1cm} (eq 5.13)

These equations describe a catalytic scheme where substrate saturation occurs. At low concentrations of substrate the initial velocity is linearly dependent on substrate. At high concentration the rate is independent of substrate. The constant $K_m$ is the substrate concentration at which the velocity is half the maximum (eq 5.14 and 5.15).
\[ \textit{k}_{\text{cat}}[E]_0 = V_{\text{max}} \]  
(eq 5.14)

\[ K_m \text{ is the } [S] \text{ at which } v = \frac{V_{\text{max}}}{2} \]  
(eq 5.15)

In some cases, \( K_m \) may then be treated as an apparent dissociation constant. It is important to remember that \( K_m \) is rate dependent and the rates are environmentally (pH, temperature, etc.) sensitive.

In our system, the consequence of being free in solution versus adsorbed to a surface may modulate an underlying intermediate states/processes. We could think about the kinetics as a series of elementary steps (eq 5.16 – 5.18). The first step does not require the further input of electrons or protons.

\[ E + H_2O_2 \stackrel{k_1}{\rightarrow} CPI + H_2O \]  
(eq 5.16)

The following elementary steps require a formal hydrogen atom donor (\( X: H \)).

\[ CPI + X: H \stackrel{k_2}{\rightarrow} CPII + X \cdot \]  
(eq 5.17)

And

\[ CPII + X: H \stackrel{k_3}{\rightarrow} E + H_2O + X \cdot \]  
(eq 5.18)

If in fact a hydrogen atom donor (or proton coupled electron transfer step) needs to be included in the \( K_m \) term the magnitude would be dependent on the concentration of donor (eq 5.19)

\[ K_m \approx \frac{k_2k_3[X:H]}{k_1(k_2+k_3)} \]  
(eq 5.19).
If in solution the donor concentration is low the $K_m$ would be repressed. If during PFV availability is no longer limiting, then $K_m$ reflects a closer representation of the actual value.

5.5 Conclusion

In this chapter we have investigated the functional role of several constitutively conserved residues within bCCPs. Trp82 functions as a conduit for electron transfer between the H- and L-hemes. Mutations to Tyr results in a drastic loss of catalytic velocity however it does not affect the affinity for hydrogen peroxide. The role of Trp61 is less clear; it is not obligatory for catalysis but could still be involved in radical exchange or the proton donation network. Globally, any mutation of these conserved Trp residues severely diminishes the activity relative to that of the wild type.

Within the L-heme pocket Gln92 and Glu102 are situated in proximity to the heme to facilitate the generation of compound I similar to the His/Arg pair found in monoheme CCPs. Gln92Asn results in a large increase to $K_m$ and modest loss of activity. This suggests that the amine side chain helps to position and direct the substrate into proper alignment within the active site. Reduction of the side chain length allows the substrate to sample more non-productive conformations. Glu102Asp shortens the side chain of a potential proton donor. This mutation corresponds to a modest increase in $K_m$, and similar in magnitude to the Trp61 mutations, however $k_{cat}$ is only reduced by half. The decrease in catalytic rate suggests an increase in the volume of the L-heme pocket.
which allows the substrate to sample additional conformations. Despite the shortening of
the side chain the distance does not drastically impair function. PFV studies of the Gln92
and Glu102 reveal a one electron reversible signal that does not correspond to $E_{\text{cat}}$ or the
potentials associated with those determined by spectroelectrochemistry. The mutations do
depress the redox potential of both hemes.

Ultimately the catalytic competence of these rNeCCP mutations makes them good
candidates for future mechanistic studies. Mutations at Q92 and E102 slow the rate of
catalysis and the rate at which protons are transferred. With these mutations we might be
able to observe a hydroperoxo intermediate which has yet to be observed for bCCPs.
Chapter 6 Stopped-flow kinetics investigation of peroxidase intermediates in *Nitrosomonas europaea* cytochrome c peroxidase

6.1 Introduction

6.1.1 Stopped-Flow spectroscopy

Analysis of transient species is complementary to investigation of steady-state parameters, potentially providing a minimal kinetic scheme, upon the observation of kinetic intermediates. In Chapter 5 we have already discussed the interpretation of kinetic constants $K_m$ and $k_{cat}$ for a bCCP enzyme and their potential complexities. It is important to remember that these steady-state values represent the limits for the rate constants associated with substrate binding, and conversion of substrate to product, *i.e.*, they alone cannot confirm a kinetically competent species, but can help to rule out possible mechanistic schemes. Stopped-flow spectroscopy is a rapid mixing technique that facilitates the investigation of transient and reactive intermediates; typically in the 1 ms – 100 s time regime. By rapidly mixing varying amounts of substrate with enzyme, transient species, complexes, and conformational changes may be observed and the associated rate constants for formation and breakdown determined through fitting mathematical models.

Limitations and complications exist within pre-steady-state analyses; foremost is observation time. After mixing the time before signal collection begins is the “dead-time,” and typically this delay is on the order of 1-4 ms in single wavelength mode, for the instrument used in this Chapter. Deconvolution of multiple species and the low
extinction coefficients of some species that should be observed add additional levels of complication, as may the role of specific buffer conditions (salt, pH, molarity) and temperature. An additional method that should be noted but will not be discussed in depth in this chapter is chemical/freeze quench-flow. For species without strong optical signals or for use of additional spectroscopies (like Mössbauer), the quench-flow apparatus mixes reactants for a fixed period of time and ultimately combine them with a quenching or freezing agent.

Typical instrumentation for stopped-flow spectroscopy incorporates the use of monochromatic light that is passed through a sample chamber and is recorded (Figure 6.1). The recorded signal is typically UV-visible absorbance, fluorescence, or circular dichroism. Absorbance may also be collected using diode array mode to rapidly collect full spectra on a rapid time scale. Each of these techniques has unique limitations, most often in terms of sensitivity and time resolution (e.g. hemes may quench fluorescence, representing a potential limit on its use, even in a stopped-flow mode.)
6.1.2 Identification of intermediates

In Chapter 4 we described the first recombinant expression system for the bacterial diheme peroxidase from *Nitrosomonas europaea* (rNe CCP). The native Ne CCP (nNe CCP) was the first bCCP to be isolated in the fully oxidized state with maximal activity, in contrast to canonical diheme peroxidases that require semi-reduction in order to allow any activity (155). Isolation of the native enzyme by Hooper *et al.* revealed several unique features with respect to the canonical bCCP enzymes (*e.g.*, Pa CCP). Hooper observed that upon pre-reduction of the H-heme steady-state activity was reduced by two orders of magnitude (41), suggesting a fundamental distinction in catalytic mechanism differentiating bCCPs into two classes: *P. aeruginosa*-like and *N. europaea*-like. A putative, minimal mechanism for Ne CCP is reproduced from Chapter 1 below as Figure 6.2 (Figure 1.12).
In the mechanism given in Figure 6.2, the initial interaction of hydrogen peroxide with the ferric heme is hypothesized to produce an iron(IV)-oxo (ferryl iron) and a cation radical intermediate. At present there has been no evidence for a hydroperoxy intermediate, preceding the production of a ferryl intermediate, like those found in eukaryotic CCPs (161, 162). The formation of an iron(IV)-oxo and cation radical species (compound I) is well documented in other monoheme enzymes: catalase-peroxidase KatG, HRP, and cytochrome P450s. Cation radicals may be stored on the heme moiety or proximal Trp residue (23), (15). Several characteristic changes of optical absorption properties have been observed for porphyrin cation radicals: reduction (or quenching) of the soret band (163), and broad increases in absorbance in the 600-700 nm region (23). In the case of cytochrome P450s the active oxidant spectrum has been reported by single-
value deconvolution and target testing which clearly illustrates differences in the active oxidant and fully ferric form (164, 165).

The soret band may be used as a primary diagnostic for the differentiation between the location of the putative cation radical (Por+• versus Trp+•) in our stopped-flow experiments. HRP and P450-like enzymes generate Fe(IV)=O Por+• as Compound I. In the cases of P450 (Figure 6.3A) and chloroperoxidase (Figure 6.3B) the generation of Fe(IV)=O Por+• causes a large drop in the ferric soret absorbance. The bleaching effect is due to the removal of an electron from the porphyrin π-system. Concurrently a peak builds in around 375 nm (Figure 6.3 panels A and B, red traces). Yeast CCP is known to generate a Fe(IV)=O Trp+• intermediate, where the porphyrin π-system remains unchanged and a red shift in the soret is observed (Figure 6.3 C). Additional features of interest are in the 500-700 nm region (Figure 6.4).

Figure 6.3 Soret features for the ferric and first intermediate species of cytochrome P450 (A), chloroperoxidase (B), and yeast CCP (C). Ferric species are all shown in black. Fe(IV)=O Por+• deconvoluted spectra for P450 (A) and chloroperoxidase (B) are shown in red. Yeast CCP (C) generates a Fe(IV)=O Trp+• intermediate which is shown by the dashed black line. The directions of change for the features are shown by the arrows. Panels (A) and (B) are from Ref (164). Data presented in panel (C) is from Ref (166).
Figure 6.4 Q band and 690 nm features for the ferric and first intermediate species of cytochrome P450 (A), chloroperoxidase (B), and yeast CCP (C). Ferric species are all shown in black. Fe(IV)=O Por +• for P450 (A) and chloroperoxidase (B) are shown in red. Yeast CCP (C) generates a Fe(IV)=O Trp +• intermediate which is shown by the dashed black line. The directions of change for the features are shown by the arrows. The high spin feature at 640 nm is denoted with an asterisk in (C), and also present in the ferric (black) species of (A). Panels (A) and (B) are from Ref (164). Data presented in panel (C) is from ref (166).

Cytochrome P450 and yCCP have the characteristic high spin features at 640 nm in their ferric forms. Upon the introduction of substrate to P450 and chloroperoxidase (Figure 6.4 A and B) a new feature at 690 nm is observed. This absorbance change is again a consequence of the generation of Fe(IV)=O Por +•; a result of removing an electron from the porphyrin π-system. Yeast CCP undergoes an increase in the Q bands (Figure 6.4 C) which is typical of an increase in electron density donated to the porphyrin π-system. In our experiments we expect to observe a quenching of the soret and a build-up of a feature in the 600-700 nm region for the generation of a Fe(IV)=O Por +•. Conversely, if Ne CCP generates an amino acid-based radical species similar to yCCP, we expect to see a red shift in the absorbance and increase in the Q bands between 500-600 nm.
In this Chapter we report on the rNe CCP peroxidase intermediates observed by stopped-flow UV-Visible absorbance spectroscopy. We have utilized both single wavelength and diode-array modes to observe spectral changes associated with the single mixing of diferric rNe CCP and hydrogen peroxide in sub-stoichiometric to pseudo-first order concentrations. Our observations support the initial hypothesis by Hooper that Ne CCP goes through a compound I like intermediate with a porphyrin cation radical.
6.2 Materials and Methods

6.2.1 Protein preparation

Expression and purification of rNe CCP was conducted as reported in Chapter 4 section 4.4.2. Oxidized and clipped rNe CCP was exchanged into 50 mM MES 50 mM HEPES pH 6.0 unless otherwise noted. Oxidation was carried out immediately before the experiment.

6.2.2 Stopped-flow spectroscopy

Transient UV/Vis kinetic studies performed with a Hi-Tech SF-61DX2 double mixing stopped-flow spectrophotometer (Salisbury, United Kingdom) in the single mixing mode. Instrument schematic is shown in Figure 6.1. The temperature was maintained by refrigerating circulator. The system was flushed with at least 100 mL 50 mM MES 50 mM HEPES pH 6.0 buffer and equilibrated to 20 °C for 10 minutes before any measurements were made. The substrate hydrogen peroxide was prepared in 50 mM 50 mM HEPES pH 6.0 and similarly equilibrated on the instrument. Time courses for experiments were recorded over the duration of 1 - 20 s for single wavelength studies. Diode array experiments were conducted on the 1 - 20 s time scale. Wavelengths were calibrated with respect to a holmium oxide glass filter at 279, 361, 454, 536, and 638 nm. Kinetic traces were fit using the software SpecFit/32 (Spectrum Software Associates).
6.3 Results

Diode array experiments were initially carried out on rNe CCP in order to acquire a broad view of the changes associated with the initial reaction with hydrogen peroxide (Figure 6.5). Overall, the diode array spectra are collected on a longer time scale due to the responsiveness of the detector. Figure 6.5 illustrates a single shot in the absence of substrate and overlaid with that of the first scan (dead time of 15 ms) with stoichiometric amounts of hydrogen peroxide. Subsequent scans are identical to that of diferric rNeCCP and omitted from Figure 6.5 for clarity. At 15 ms we have observed an overall loss of intensity at 408 nm and a concurrent increase at 675 nm. Therefore, the majority of the spectral changes take place over approximately 50 ms under these conditions. The reduction (or bleaching) at 408 nm and increase at 675 nm suggest the formation of Fe(IV)=O Por** similar to that observed in HRP and P450-like enzymes (Figure 6.3 and Figure 6.4).
Figure 6.5 Oxidized rNe CCP in the absence of peroxide collected in diode array mode (black). 15 ms diode array spectrum of 2 µM Ne CCP with 2 µM hydrogen peroxide (red) in 5mM HEPES 5mM MES 10 mM NaCl 1 mM CaCl₂ pH 6.0 at 20 °C.

Observation of the feature at 675 nm has proved difficult to reproduce reliably under the low ionic-strength conditions. One issue common for transient techniques is the sensitivity to the ionic strength. From here many conditions were explored. For single wavelength experiments 50 mM MES 50 mM HEPES provided the most reproducible data at pH 6.0. Single wavelength studies were conducted at the soret maxima 408 nm, the maxima of the semi-reduced and diode array maxima 418 nm, and at 675 nm. Of all the collected data (pH 6, 20 °C) those collected at 408 nm are the most reproducible.

Stopped-flow spectroscopy at a single wavelength enables observations to be acquired much more rapidly than with diode array spectroscopy. The dead time for our instrument is approximately 3 ms. In our hands the most reproducible data was collected at 408 nm. A single shot in buffer shows very little variation over 1 second (Figure 6.6).
Figure 6.6 Single wavelength trace at 408 nm of 4 µM Ne CCP in the absence of hydrogen peroxide in 50 mM MES 50 mM HEPES pH 6.0

Figure 6.7 Reactions of 4 µM Ne CCP with varying amounts of hydrogen peroxide collected at 408 nm: (A) 1 µM, (B) 8 µM, and (C) 40 µM hydrogen peroxide. At sub-stoichiometric amounts hydrogen peroxide (A) the beaching is apparent by the appearance of a minima at ~12-15 ms and recovery at ~175 ms. At approximately stoichiometric concentration there is an observed increase in intensity before settling (B). At gross excess substrate the increase before final recovery is suppressed. Fit to a three exponential scheme $A \rightarrow B \rightarrow C \rightarrow D$ (Red).
The response at 408 nm changed slightly as a function of peroxide concentration. At peroxide concentrations near and below stoichiometric amounts, a clear quenching of the signal and recovery was observed (Figure 6.7A). Upon increasing peroxide, directly observing the reduction of signal at 408 nm or “quench,” is impossible in real-time (Figure 6.7 A vs B and C), and even the apparent recovery in A (2 - 15 ms) is harder to discern.

Dolphin reported that as a consequence of removing an electron from the porphyrin π system in catalase and HRP a concurrent loss of absorbance in the soret and an increase in the 600-700 nm region is observed. Recovery of the soret is a function of
reducing the radical (163) (Figure 6.8). In P450s compound I may be prepared by reaction with m-chlorobenzoic acid Figure 6.3 (A and B) which also behave in a similar manner as we observer 408 nm (quench and recovery to approximately starting absorbance).

![Figure 6.9](image)

Figure 6.9 Single wavelength trace at 408 nm of 4 µM Ne CCP with 8 µM hydrogen peroxide 50 mM MES 50 mM HEPES pH 6.0 (Black). (A) Fit to a two exponential scheme $A \rightarrow B \rightarrow C$ (Red). (B) Fit to a three exponential scheme $A \rightarrow B \rightarrow C \rightarrow D$ (Red).

The development of a kinetic model was first done through fitting of the single wavelength data. The first scheme used was a two exponential fit $A \rightarrow B \rightarrow C$ where A is the diferric form, B is the first kinetic intermediate, and C the product. This scheme
agrees with the bulk of the data but clear deviations occur at the top of the recovery phase (25-50 ms), as shown using the 1:2 CCP:substrate data reproduced in Figure 6.9. The addition of a fourth species, producing an $A \rightarrow B \rightarrow C \rightarrow D$ model provides a much more complete agreement with the experimental data as shown through examination of the residual absorbance. The inclusion of additional parameters comes with the caveat of better fit to experimental data. In our case the addition of a third parameter makes chemical sense. Inclusion of four or more parameters results in fits that fail, or produce errors far exceeding the calculated rate constant. Comparisons of the two and three exponential fits are shown in Figure 6.9. The resulting rate constants, determined as a function of substrate concentration are plotted in Figure 6.10 and are given in Table 6.1.

![Figure 6.10](image)

Figure 6.10 (A) Plot of $K_1$ (■) and $K_3$ (●) as a function of hydrogen peroxide concentration. (B) Plot of $k_1$ (■), $k_2$ (▲), and $k_3$ (●) as a function of hydrogen peroxide concentration. $k_2$ and $k_3$ are essentially independent of hydrogen peroxide. Spectral changes observed at 408 nm. The data in panel (A) is fit to a two exponential fit scheme $A \rightarrow B \rightarrow C$. Panel (B) is fit three a three exponential scheme $A \rightarrow B \rightarrow C \rightarrow D$. All experiments were used with 4 µM rNe CCP. The variation of $k_3$ is so small they look like a single point in these plots. Kinetic parameters are summarized in Table 6.1.
The first step in both schemes ($k_1$, black squares) is peroxide dependent, and linear with respect to peroxide generating a second order rate constant around $10^6$ M$^{-1}$ s$^{-1}$. For the kinetic model $A \rightarrow B \rightarrow C$, $k_3$ is independent of peroxide, slow, and identical to the slow step of the other model. The second model, $A \rightarrow B \rightarrow C \rightarrow D$, incorporates step $k_2$ (Figure 6.10B, blue triangles) which is also peroxide independent but approximately 12.5 times faster than $k_3$. The three exponential fits give rise to build-up curves that are consistent with the fast generation of the first intermediate species in the first 2-25 ms, a second intermediate in the 25-75 ms regime, and decay to a final species.

Table 6.1 Summary of rate constants associated with the single turnover of diferric Ne CCP in the presence of hydrogen peroxide.

<table>
<thead>
<tr>
<th>Model</th>
<th>$k_1$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$k_3$ (s$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>ABC</td>
<td>$3.20 \times 10^6$</td>
<td>-</td>
<td>$4.5 \pm 0.15$</td>
</tr>
<tr>
<td>ABCD</td>
<td>$1.60 \times 10^6$</td>
<td>$50 \pm 3$</td>
<td>$4.1 \pm 0.13$</td>
</tr>
</tbody>
</table>
Figure 6.11 Concentration build-up curves for increasing concentrations of substrate reacting fit to three exponential scheme $A \rightarrow B \rightarrow C \rightarrow D$ with 4 µM rNe CCP 50 mM MES 50 mM HEPES pH 6.0. (A) 8 µM hydrogen peroxide (B) with 20 µM hydrogen peroxide (C) with 40 µM hydrogen peroxide (D) with 60 µM hydrogen peroxide.
6.4 Discussion

The major distinguishing factor of Ne CCP with respect to other bacterial diheme peroxidases is that it is fully active in the diferric form (41). In other bCCPs the H-heme is pre-reduced and poised to shuttle an electron to the L-heme upon interaction with hydrogen peroxide (155). This difference suggests that Ne CCP behaves mechanistically much more like traditional monoheme peroxidases (HRP and yCCP in addition to heme enzymes that activate oxygen in a manner similar to cytochrome P450s (15). These enzymes have been shown to undergo a two electron oxidation and to form an iron(IV)-oxo species and a radical (169). In all these cases except for yCCP this intermediate is a porphyrin cation radical. In the case of yCCP the radical is housed on Trp191 (169). In Chapter 5 we described several mutations to conserved tryptophan residues. Trp82 functions as a conduit from the H-heme to L-heme. Trp61 is conserved within Loop-1 and mutations Trp61Tyr and Trp61Phe slow activity considerably but does not abolish activity. Furthermore, drastic changes to substrate affinity occur with these substitutions. Considering the current minimal mechanistic model (Figure 6.2) and steady-state kinetic studies in Chapter 5, it is likely that if a radical is generated, it would be porphyrin based.

Porphyrin radicals have been well characterized in cytochrome P450s, HRP and yCCP mutants, but Ne CCP provides a unique challenge not present in the monoheme enzymes. The second heme moiety further complicates the UV/Vis spectrum not allowing for direct comparison of well-characterized and relatively long-lived intermediates. Now that we have available recombinant system for overexpression of Ne
CCP we are able to pursue more protein intensive techniques such as stopped-flow spectroscopy.

Several pieces of spectroscopic evidence support the formation of a porphyrin cation during the initial interaction with rNe CCP. During the early 1970s, Dolphin and coworkers demonstrated that a reduction or quenching of the soret band was a result of the removal of an electron from the porphyrin $\pi$ system of both catalase and HRP (163, 167). Erman has shown that upon the initial reaction of yeast cytochrome $c$ peroxidase where Trp191 has been mutated to phenylalanine, an initial bleaching at 416 nm occurs and is again indicative of porphyrin radical formation. (Subsequent transformations of the first intermediate are then attributed to cation radical reduction.) These species were also observed approximately within the first 15 ms (169, 170). We clearly observe a rapid reduction of the soret at 408 at low substrate concentrations. The loss at 408 is substrate dependent and eventually becomes too fast to observe directly, as the substrate concentration is increased (Figure 6.7). The deconvoluted spectrum of cytochrome P450 and chloroperoxidase compound I have several key spectroscopic features that are similar to those observed for Ne CCP. A peak appears at ~675 nm upon introduction of substrate and the soret feature is reduced while an additional feature grows in that is blue shifted (Figure 6.3 and Figure 6.4.) (164). HRP compound I has a strong absorbance at 646 nm and a quenching of the absorbance at 400 nm (171). Our diode array spectra clearly shows an increase at 675 nm and a quenching of the soret (Figure 6.5) In general the increase in absorbance in the 600-700 nm region has been attributed to build up of the
Fe(IV)=O Por⁺. During many of the lower substrate concentrations the initial bleaching and recovery at 408 nm is consistent with the similar behavior of yCCP Tyr191Phe which would correspond to the reduction of the radical species (Figure 6.8). This does not rule out the possibility that the conserved Tyr may play a secondary/back-up role for radical storage, or as an acceptor when reducing equivalents are not readily available.

In contrast to those features reported for Compound I for HPR and P-450s, we would expect very small changes in the soret accompanied by changes around the Q bands (500-600 nm). These have been demonstrated in the literature for yCCP and Pa CCP (24, 157), and for the So CCP (thesis work of Dr. Goçke Su Pulcu).

Figure 6.7B clearly shows the generation of a local maximum at ~ 25 ms during the initial fast phase during the oxidation of hydrogen peroxide. The origins of this maximum may be explained by analogy myeloperoxidase/chloroperoxidase and Pa CCP like bCCPs, where enhanced soret intensity has been attributed to a ferryl iron (iron(IV)-oxo) following reduction of Compound I. Myeloperoxidase (172) and chloroperoxidase (173) are P450-like enzymes that can catalyze the oxidation of chloride to hypochlorite through the generation of a Compound I species (174). Cong and coworkers have synthesized a variety of heme model complexes that stability bind hypochlorite at -60 (175-177). The initial complex has a soret maxima around 403 nm. Upon warming they slowly decompose to an iron(IV)-oxo species with Soret maxima at 414 nm and a broad visible band at 546 nm. The Soret increases in intensity by approximately one third (175).
Similarly, Dunford described compound II formation for the Pa CCP with a soret maxima at 420 nm (178).

Two models have been put forth through the kinetic fitting of data associated with spectral changes at 408 nm. Common among the two models are a fast, peroxide-dependent $k_1$ with an apparent second-order rate constant of approximately $10^6$ M$^{-1}$ s$^{-1}$. This is slightly slower than reported for yCCP (174) and for So CCP (157), where the analogous value is $\sim 10^7$ M$^{-1}$ s$^{-1}$. Using the concentration profiles of Figure 6.11 and the spectral properties suggestive of intermediates, including a porphyrin cation radical and a species resembling compound II, we propose the mechanisms in Figure 6.12 and Figure 6.13. In the first step the ferric enzyme (species A) and substrate interact to form compound I (species B), while potentially going through a hydroperoxo intermediate that is facilitated by the acid base properties of Glu92 and Gln102. This process is fast, and the intermediate reaches its maximal population within $\sim 25$ ms, and the processes would give rise to the rapid H$_2$O$_2$-dependent soret bleaching phase that we have observed. Subsequent recovery of Soret intensity would be anticipated in the following H$_2$O$_2$-independent steps. In a proposed first step, the putative Por$^{++}$ species may be reduced by one electron, regenerating neutral Por within species C with similar Soret absorption characteristics to the fully oxidized enzyme; subsequent reduction of the ferryl species likely follows, a step that we hypothesize would be relatively slow (and likely proton-coupled) producing species D. We note that the identity of a potential electron donor to species B is not included in our studies: no additional source of electrons should be
accessible in our experiments here. While reducing equivalents would be available from cytochrome $c$ in steady-state kinetics, here the source for electrons in the proposed mechanism is enigmatic, as the enzyme has been poised in the fully oxidized state. Possible sources of the reducing equivalents required to reduce the presumed species B include: nearby Trp residues, which could be oxidized to a Trp$^{+\bullet}$ radical (Figure 6.12) or the Fe(III) ion of the high-potential heme (Figure 6.13). The conserved Trp61 found in proximity to the active site may be a safety mechanism for Ne CCP to displace a highly reactive iron(IV)oxo. Finally, Figure 6.14 depicts a P450-like mechanism that could take place where the generation of compound I is the same as previously, but later steps would be result in a rebound mechanism in the absence of additional reducing equivalents. In such a mechanism hydrogen atom abstraction occurs from Trp61 leaving behind a cation radical. The hydroxyl group could rebound to Trp61 on loop 1, and the Fe(IV) is reduced to Fe(III).

Ultimately the nature of the kinetic species has yet to be definitively identified. The build-up curves (Figure 6.11) and mechanistic hypotheses (Figures 6.12 - 6.14) suggest possible states to be trapped and probed by alternative spectroscopies (like Mössbauer and EPR). All of the mechanistic models involve the production of compound I intermediate which is likely the first observable one here at approximately 12 ms. Interrogation of this species would probably best be performed using rapid freeze quench EPR. If we were to generate a Trp$^{+\bullet}$ radical (Figure 6.12 and 6.14) these would appear later based on build-up curves and proposed mechanisms occurring between 50-150 ms.
We would expect to observe EPR signals similar to those of ribonucleotide reductase (15).

Figure 6.12 Mechanism 1 proposed for the interaction of diferric Ne CCP with hydrogen peroxide based on kinetic observations and the fitting of a three step model. R is likely Trp61 on loop 1.
Figure 6.13 Mechanism 2 proposed for the interaction of diferric Ne CCP with hydrogen peroxide based on kinetic observations and the fitting of a three step model.
Figure 6.14 Mechanism 3 proposed for the interaction of diferric Ne CCP with hydrogen peroxide based on kinetic observations and the fitting of a three step model. R is likely Trp61 on loop 1.
6.5 Conclusion

This chapter presents the initial transient kinetic characterization of rNe CCP with hydrogen peroxide. The vast majority porphyrin-oxo intermediates within the literature are for monoheme peroxidases and P450 like enzymes. However the spectroscopic signatures present for those enzymes have helped us to develop the foundation for kinetic models discussed here (Figure 6.12 and Figure 6.13 and Figure 6.14). Diode array and single wavelength data support the production of a cation radical within the first 15 ms. Further, the single wavelength data is indicative of a compound II or III like species that then slowly decays. Further studies are continuing to probe the pH dependence of the wild type enzyme. In Chapter 5 we have shown that Gln92 and Glu102 are important in the catalytic cycle and likely take part as acid/base partners during the formation of compound I. We are investigating how these mutations are involved in peroxide activation. These mutations in addition to the Trp mutants will be valuable in further studies to characterize these intermediate species.
Appendix A: Hybrid cluster proteins from *Escherichia coli* K12 and *Shewanella oneidensis*

*A.1 Introduction*

Hybrid-cluster proteins (HCP), formerly ‘prismane proteins’, were initially purified as a contaminant during the isolation of the hydrogenase from *Desulfovibrio vulgaris* Hildenborough (Dv) (179). HCPs are widely distributed among bacteria, archaea, and single celled eukaryotes, but primarily through facultative and strict anaerobic bacteria. The hybrid cluster binding motif allows for the differentiation between HCPs into three sub-classes (Table A1). Class I and II HCPs also possess a helical repeat at the c-terminus.

<table>
<thead>
<tr>
<th>Class</th>
<th>Bacterial Source</th>
<th>Hybrid Cluster Binding Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Strictly anaerobic bacteria and methanogenic archaea</td>
<td>Cys-(X&lt;sub&gt;aa&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;-Cys-(X&lt;sub&gt;aa&lt;/sub&gt;)&lt;sub&gt;7-8&lt;/sub&gt;-Cys-(X&lt;sub&gt;aa&lt;/sub&gt;)&lt;sub&gt;5&lt;/sub&gt;-Cys</td>
</tr>
<tr>
<td>II&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Anaerobic gram-negative bacteria</td>
<td>Cys-(X&lt;sub&gt;aa&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;-Cys-(X&lt;sub&gt;aa&lt;/sub&gt;)&lt;sub&gt;11&lt;/sub&gt;-Cys-(X&lt;sub&gt;aa&lt;/sub&gt;)&lt;sub&gt;6&lt;/sub&gt;-Cys</td>
</tr>
<tr>
<td>III&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Hyper-thermophilic bacteria and archaean</td>
<td>Cys-(X&lt;sub&gt;aa&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;-Cys-(X&lt;sub&gt;aa&lt;/sub&gt;)&lt;sub&gt;7-8&lt;/sub&gt;-Cys-(X&lt;sub&gt;aa&lt;/sub&gt;)&lt;sub&gt;5&lt;/sub&gt;-Cys</td>
</tr>
</tbody>
</table>

Table A1 Classification of HCPs by sequence condensed from van den Berg et al (180).<sup>a</sup>Class II HCPs have a putative reductase immediately downstream. <sup>b</sup>Class 3 HCPs are similar to class 1, but contain a 116 residue deletion preceding the hybrid cluster (180).

Biochemical (181-190) and bioinformatic (191, 192) studies (Figure A1) have revealed that HCPs are a member of a wider family of iron-sulfur proteins. The HCP-like superfamily (NCBI CDD cd00587; NCBI conserved domain database) also includes acetyl-CoA synthase (ACS), and carbon monoxide dehydrogenase (CODH) (193). The conserved domain cd00587 is illustrated in Figure A1.
On the basis of the initial EPR spectra a hypothesis was put forth that the enzyme contained an iron-sulfur cluster with a novel $[6\text{Fe}-6\text{S}]$ core. Shortly after the initial report, Moura et al. isolated a similar enzyme form *Desulfovibrio desulfuricans* (Dd) with similar spectroscopic signatures (195). Extensive biophysical characterization and comparison with $[6\text{Fe}-6\text{S}]^{3+}$ model compounds supported the initial spectroscopic assignment (196-200). It was not until the late 1990s that a crystallographic study of the so-called “hybrid cluster protein” revealed the presence of two iron-sulfur clusters.
Cluster 1 represents a typical [4Fe-4S] cluster, Cluster 2 is a novel [4Fe-2S-2O] cluster with μ-oxo bridges (201).

Correlation of the retention of HCP in micro-organisms is strongly correleated with gene products necessary for inorganic nitrogen metabolism, leading to the suggestion that HCP itself may react with hydroxylamine or nitric oxide (191, 192, 202), two toxic intermediates in the reduction of nitrite during ammonification. Several detoxification pathways have been established for response to NO: flavohemoglobin (hmp) and the flavorubredoxin (norVW) (203-205). The potential role of HCP in hydroxylamine detoxification was established by its reductase activity in E. coli (206) and in vivo activity in R. capsulatus (207). HCP is regulated by several nitrogen-specific transcription factors.
of the CRP/FNR family which regulate respiratory nitrite reductase (nar) and ammonifying operons (nir and nrf). In some genomes a HCP specific regulator (HcpR) is present (191, 192, 208). The hypothesized role of HCP in the inorganic nitrogen cycle has been summarized in Figure A3.

![Figure A3. The Bacterial Inorganic Nitrogen Cycle. Solid lines indicate enzymatic transformation. The black dashed line indicates a non-enzymatic pathway for the conversion of nitric oxide to hydroxylamine. Dotted blue lines indicated intermediate release from cytochrome c nitrite reductase. Adapted from Rodionov et al. (192) and used without permission.]

Given the potential connection to nitrogen metabolism, typically hydroxylamine reductase activity has been considered in the reported purifications of HCP enzymes, with steady state kinetic parameters giving a wide-range of catalytic efficiencies (i.e., $k_{cat}/K_m$ for NH$_2$OH is reported from $10^5$-$10^7$ M$^{-1}$s$^{-1}$, as summarized in Table A2). Notably, none of the catalytic measurements were conducted with the native electron donor, a hybrid cluster protein reductase (HCR) that is found in the same operon as the hcp gene.
Class II HCPs have a reductase immediately downstream of the HCP gene (209). HCR is hypothesized to be a NAD(P)H oxidoreductase containing a flavin and a [4Fe-4S] cluster.

In the presence of reduced reductase, reduction of fully oxidized *E. coli* HCP was too fast to measure through manual mixing (209).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Class</th>
<th>Substrate</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. vulgaris</em></td>
<td>I</td>
<td>NH$_2$OH</td>
<td>3.1</td>
<td>NR</td>
<td>NR</td>
<td>211</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>0.04</td>
<td>0.3</td>
<td>0.1 x 10$^3$</td>
<td>210</td>
</tr>
<tr>
<td><em>R. capsulatus</em></td>
<td>II</td>
<td>NH$_2$OH</td>
<td>1.9</td>
<td>1</td>
<td>1.9 x 10$^3$</td>
<td>181</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>E. coli</em></td>
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<td>NH$_2$OH</td>
<td>396</td>
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<td>1.59 x 10$^5$</td>
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<td>H$_2$O$_2$</td>
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<td>0.3</td>
<td>0.6 x 10$^3$</td>
<td>211</td>
</tr>
<tr>
<td><em>P. furiosus</em></td>
<td>III</td>
<td>NH$_2$OH</td>
<td>3.8</td>
<td>0.4</td>
<td>9.5 x 10$^3$</td>
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<td></td>
<td></td>
<td>H$_2$O$_2$</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

Table A2. Kinetic parameters of hydroxylamine and hydrogen peroxide by HCP (206, 210-212). For *R. capsulatus* and *P. furiosus* the kinetics utilizing peroxide as a substrate were not examined.

In this Appendix we describe our effort to clone and express two HCPs and their respective reductases from *E. coli* and *S. oneidensis*. Our goals were to study the steady state kinetics using the native electron donor and investigating the mechanism of how HCP does multi-electron chemistry through protein film voltammetry.

### A.2 Materials and Methods

#### A.2.1 Constructs and cloning

The genes encoding the *E. coli* HCP (b0873) and putative NAD(P)H reductase (b0872) were amplified by PCR from *E. coli* K12 genomic DNA. Primers 5’-
AATTAAGGTCTCCTCCTAAATGGTTTGTGTGCAATGTGAACAAAC-3’ and 5’-
AATTAAGGTCTCAGCGCTTTACGCGCTCAACAGTTGC-3’ were used for amplification of the \( \text{Ec} \) HCP. Primers 5’-
AATTAATGACGATGCCAACGAATCAATG-3’ and 5’-
AATTATTATGCGAGAACCAAATCC-3’ were used for \( \text{Ec} \) HCR. The inserts and pASK-IBA3+ (IBA-Life Sciences) were digested with BsaI and then ligated with T4 DNA ligase for 16 hours at 4 °C to yield pEC-HCP006 and pEC-HCR003. The amino acid and nucleotide sequences are shown in Figure A4 Figure A5.

We also received the plasmids pWB208 and pWB302 \( (180) \) as generous gifts from Walter van Dongen. The pWB208 contained a 2.8 kb insertion containing both the \( E. \text{coli} \) HCP and HCR inserted between BamHI and KpnI in the pUC18 subcloning vector. pWB302 contains the HCR gene inserted into the expression vector pTYB11 (NEB) which expresses a chitin binding domain fusion as part of the IMPACT™-CN system (NEB). Over production of these enzymes were pursued as previously reported \( (180) \) however no expression was observed.

The genes encoding the \( S. \text{oneidensis} \) HCP (SO1363) and putative NAD(P)H-dependant HCP reductase (SO1364) were amplified by PCR from \( S. \text{oneidensis} \) MR-1 genomic DNA. Primers 5’-
AATTAAGGTCTCCTCAAATGTATGTGTTCTGTATTCCAGTGGAGCA-3’ and 5’-
AATTAAGGTCTCAGCGCTCTGCTCTAAGCCACGTTCAGCATG-3 were used for amplification of the \( \text{So} \) HCP. Primers 5’-
AATTAAGGTCTCAAATGAAGTTTGATTATAAGCCCGGTC-3’ and 5’-
AATTAAGGTCTCAGCGCTGATCAACGAAAGCGTCACAT-3’ were used for So HCR. The resulting constructs are pSO-HCP004 and pSO-HCR003 with pASK-IBA3+ as the parent vector. The amino acid and nucleotide sequences are shown in Figure A6 and Figure A7.
**E. coli** hybrid cluster protein

Amino acid sequence

MFCVQCEQTIRTPAGNGCSYAQGMCGKTAETSDLQDLLIAALQGLSAWAVKAREYGIINH
DVSDLGELQRQAETPKDNAIGENILRLRLCLYLGLGAAAYMEAHVILQYNDIY
AQYHKIWMALGTPADMMALECSMEIQGNNFVKMSILDAGETGKYGHPTPTQVNVKATA
GKCILISGHDKLKDLYNLEQTEGTGVNVYTHGEMLPAGYEPRLRKFKHLVGVNYGSGWQYQ
QVEFAPFPGPIVMTSNCIIPTVGAYDRIRWIVSTGVFGVHRDLDGDFSATVTQAAQMA
GFYSEIPHLTVGFGRQTLGLAADTLIDLVSREKLRHIFLLGGCDGAGERHYFTDFAT
SVPDDLILTLACGKYRENKLEFGDIEGLRLVPDLAQAQCNDAISAILVTLAEKLGCGVN
DLPLSLVLSWFEQKAIVILTTLSLGVKIVTGPTAPGLTLPDLAVLENEKFLRSITTV
EEDMKQLLSA

Nucleotide sequence

atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc
ggcctgtggcgctgcaataaacaccgccctgctgctgctgagattaccaacctcgcgc
acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc
ggcctgtggcgctgcaataaacaccgccctgctgctgctgagattaccaacctcgcgc
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acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
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gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc
ggcctgtggcgctgcaataaacaccgccctgctgctgctgagattaccaacctcgcgc
acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc
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acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc
ggcctgtggcgctgcaataaacaccgccctgctgctgctgagattaccaacctcgcgc
acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc
ggcctgtggcgctgcaataaacaccgccctgctgctgctgagattaccaacctcgcgc
acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc
ggcctgtggcgctgcaataaacaccgccctgctgctgctgagattaccaacctcgcgc
acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc
ggcctgtggcgctgcaataaacaccgccctgctgctgctgagattaccaacctcgcgc
acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc
ggcctgtggcgctgcaataaacaccgccctgctgctgctgagattaccaacctcgcgc
acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc

tttaaacaggttctgctgctgagattaccaacctcgcgc
acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc

tttaaacaggttctgctgctgagattaccaacctcgcgc
acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc

tttaaacaggttctgctgctgagattaccaacctcgcgc
acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc

tttaaacaggttctgctgctgagattaccaacctcgcgc
acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc

tttaaacaggttctgctgctgagattaccaacctcgcgc
acctgttgtgagattaccaacctcgcgc
atgttttgtgtgcctaagtgtgaacaacactatccgtactccggcaggaacgcgtctcatac
gcgccagggctgttgattataacccggcagaaacctctgctgctgagattaccaacctcgcgc

Figure A4. Amino acid and nucleotide sequence for *E. coli* hybrid cluster protein (HCP).
**E. coli hybrid cluster reductase**

**Amino acid sequence**
MTMPTNQCPWRMQVHHTQETPDWTISLICHYYPYRAGQYALVSVRNSAETLRA

**Nucleotide sequence**

```
E. coli hybrid cluster reductase

Amino acid sequence
MTMPTNQCPWRMQVHHTQETPDWTISLICHYYPYRAGQYALVSVRN

Nucleotide sequence
atgacgatgccaacgaatcaatgcccgtggcggatgcaggttcatcacattacgc
```

**Figure A5. Amino acid and nucleotide sequence for E. coli hybrid cluster reductase (HCR).**
**Amino acid sequence**

MFCIQCEQTI RTPA NGC SYA QMG C GLA ATSDLQ DLL YMLQ GVS VYAVK RE LG VD TVE DTF VP KAF
FSTLTN VNF DDE RII AYA QAA QYRES LKN YAET CEQ S GKT AE MQP VAQL VG TS KLE ML SQAP ILS
NKG KNNI HED L GL RL LC Y LGK KAA Y MEHAR LV GRTVD DI 1 AADF HRIMA FL GPE SVADA KLF S TAMEI
GQI NYRM AAL DAE TGA E FH I PE TVN TKP VKG AIL VSG HD MK D LE L E Q T AK G IN YTH GE ML PA
L AYP AF KKY PHL VVY GY S A W OQ KFE KF A N P G AV MT S NC ID IP V Q Y S DR I FR S I VG P G V H V TD
D FSV VIE KAL S N D G FY E D I P H INT G F A R N A L M A A P T V VEN KNS I KHF FL VGG CD G D K ER S Y TD
L AK S P K DS II L T L G CK Y K PK NL F D G IN I P R L D I G Q N D A Y S AI Q IA L S Q IF ECD IN E L P L NL V
LS WFE Q KAI V V L L L S LG V NK I R T G P A P A F LT A N L K I L E D K F L R N T V T E A DL K T ML N V A

**Nucleotide sequence**

ATG TTCT CGT AT TCC AGT GTG AG CAA ACT TAT TCG TAT AT CCAG CCG GCA ACG GTT GAT GCTAT GCG CA AAG G TA
TG TGT GGCA AAT TGG CCG CCG ACC TCCG AT TCGA AAG ACT TAT TAC TAT GCT GCA GGG GTT TT CAG T
GT ATG CCG TAA AAA AGC CCG GCA GCT AGG TGT GGA TAC A GAG TAT ACC TAT TGT GAC AAG GAT TCA
TT CCG ACC TTA ACC AAG CTA GAA GGT TAT CCG GAC AAG GGT GAT GCC AGC TAC ACC CTT AAG GCT GGA CAG
CC ACG TCC GAT GCT GTC TAC TGC AAT TTT TTA AAC CGT GAC CAA GGT TAT CCG CTT AAT ATT CTT AAT CTT
AAT CTT AAT CTT TTA AAA AAT TAC CCA TTA TGA ATG GTA ACT AC GGG CAG TTA AAC CC
AAA GAA TTT CCC TAT TGG GAC AAT TGG GAA AAC CCC TAA AAG TAA AAC GCA TTA TTA AAG TTA AAT TTA
GCA TAT TAA GCT TCT GCT CGT TGG TAA CAA GGA ACT TAC ATG GTA ACT AC GGG CAA TAT TTA
GAC AGT TGG TAA GCT TTA AAG TAA AAC GCA TTA TTA AAG TTA AAT TTA GCA TAT TTA AAG TTA AAT TTA
GCA TAT TAA GCT TCT GCT CGT TGG TAA CAA GGA ACT TAC ATG GTA ACT AC GGG CAA TAT TTA
GAC AGT TGG TAA GCT TTA AAG TAA AAC GCA TTA TTA AAG TTA AAT TTA GCA TAT TTA AAG TTA AAT TTA
GCA TAT TAA GCT TCT GCT CGT TGG TAA CAA GGA ACT TAC ATG GTA ACT AC GGG CAA TAT TTA
GAC AGT TGG TAA GCT TTA AAG TAA AAC GCA TTA TTA AAG TTA AAT TTA GCA TAT TTA AAG TTA AAT TTA
GCA TAT TAA GCT TCT GCT CGT TGG TAA CAA GGA ACT TAC ATG GTA ACT AC GGG CAA TAT TTA
GAC AGT TGG TAA GCT TTA AAG TAA AAC GCA TTA TTA AAG TTA AAT TTA GCA TAT TTA AAG TTA AAT TTA
GCA TAT TAA GCT TCT GCT CGT TGG TAA CAA GGA ACT TAC ATG GTA ACT AC GGG CAA TAT TTA
GAC AGT TGG TAA GCT TTA AAG TAA AAC GCA TTA TTA AAG TTA AAT TTA GCA TAT TTA AAG TTA AAT TTA
GCA TAT TAA GCT TCT GCT CGT TGG TAA CAA GGA ACT TAC ATG GTA ACT AC GGG CAA TAT TTA
GAC AGT TGG TAA GCT TTA AAG TAA AAC GCA TTA TTA AAG TTA AAT TTA GCA TAT TTA AAG TTA AAT TTA
GCA TAT TAA GCT TCT GCT CGT TGG TAA CAA GGA ACT TAC ATG GTA ACT AC GGG CAA TAT TTA
GAC AGT TGG TAA GCT TTA AAG TAA AAC GCA TTA TTA AAG TTA AAT TTA GCA TAT TTA AAG TTA AAT TTA
GCA TAT TAA GCT TCT GCT CGT TGG TAA CAA GGA ACT TAC ATG GTA ACT AC GGG CAA TAT TTA
GAC AGT TGG TAA GCT TTA AAG TAA AAC GCA TTA TTA AAG TTA AAT TTA GCA TAT TTA AAG TTA AAT TTA
GCA TAT TAA GCT TCT GCT CGT TGG TAA CAA GGA ACT TAC ATG GTA ACT AC GGG CAA TAT TTA
GAC AGT TGG TAA GCT TTA AAG TAA AAC GCA TTA TTA AAG TTA AAT TTA GCA TAT TTA AAG TTA AAT TTA
GCA TAT TAA GCT TCT GCT CGT TGG TAA CAA GGA ACT TAC ATG GTA ACT AC GGG CAA TAT TTA
GAC AGT TGG TAA GCT TTA AAG TAA AAC GCA TTA TTA AAG TTA AAT TTA GCA TAT TTA AAG TTA AAT TTA
GCA TAT TAA GCT TCT GCT CGT TGG TAA CAA GGA ACT TAC ATG GTA ACT AC GGG CAA TAT TTA
GAC AGT TGG TAA GCT TTA AAG TAA AAC GCA TTA TTA AAG TTA AAT TTA GCA TAT TTA AAG TTA AAT TTA
GCA TAT TAA GCT TCT GCT CGT TGG TAA CAA GGA ACT TAC ATG GTA ACT AC GGG CAA TAT TTA
GAC AGT TGG TAA GCT TTA AAG TAA AAC GCA TTA TTA AAG TTA AAT TTA GCA TAT TTA AAG TTA AAT TTA

**Figure A6. Amino acid and nucleotide sequence for S. onidensis hybrid cluster protein (HCP).**
**S. oneidensis** hybrid cluster reductase

**Amino acid sequence**
MQNIMPNTLSSTPANPVGFNQLCVERWHEDAVSSFRFQAGEPMFKEYKPGQFITFVL
EINGEACRSYTLSTPSRPYSMLVTKRDGLGIVSNYLIDHELQGVTTLQRTQPT
FDIAKYLFLSAGGIGTIpMSRMLDQ1INADIALVSHARTQAD1IFKNTLETMAAR
HASFKLCYLVEGVTDDTVHWEAEFAHYVGLSQAQNLSLVPDFAEIVFLOCGPELYMQAV
KTILTELNFDMKNLYHESFATAVKEAQSHVKQAEIQSETPTTSTSTGFMLSIGDKKHLLTA
EQTLLDGIEAEGLPIIAACRSGVCACKVLQGETESTSMTLPTDIEAGYVLACSTR
LKSDVTLSLI

**Nucleotide sequence**
atgcactattcatcagcactgcttttatctctacgcccgaattcctttgggttt
aaccgtagagtctggtggagcgtgctgcagcactagtgatttctttaaaccgactctcat
ctccgagctgggtaaggttctatttaacctgcaatcctgtttgctatttcctcttctc
caagcaggtgagccgatgtggtgatggtggtgctagttttctcctctctctctctctc
tactcttatttgctagattatagcgggtgtttgttttctctctctctctctctctct
ctattcattcataacagtggcagcactacgtttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
A.2.2 Hybrid cluster protein and hybrid cluster reductase expression

HCPs from both *E. coli* and *S. oneidensis* were transformed into chemically competent cells (BL21(DE3), Rosetta, or JM109) without and bearing the pDB1281 containing the *isc* operon for optimal FeS cluster biosynethesis. Pre-cultures (5 mL) were grown overnight in LB medium. Large cultures were inoculated with a 1:100 dilution and incubated at 37 °C, 200 rpm, until OD$_{600}$ = 0.2. L-arabinose (sigma) was added to 0.2 g/L to induce pDB1281. Growth was continued until OD$_{600}$ = 0.5. The cultures were removed from the incubators and allowed to reach room temperature. Cultures were induced with 75 µg/L IPTG and allowed grow on the bench top overnight. All media was supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin for cells containing pDB1281. Cells were harvested by centrifugation (8000 x g, 4 °C, 15 min) and pellets were frozen at -20 °C.

HCRs were expressed in JM109 or BL21(DE3) cells with and without the pDB1281 vector. 5 mL pre-cultures were grown overnight in LB medium. Large cultures were inoculated with a 1:100 dilution and incubated at 37 °C, 200 rpm, until OD$_{600}$ = 0.5 and induced with 75 µg/L IPTG for 6 hours. All media was supplemented with 100 µg/mL ampicillin and 50 µg/mL kanamycin for cells containing pDB1281. Cells were harvested by centrifugation (8000 x g, 4 °C, 15 min) and pellets were frozen at -20 °C.

Cells were thawed at room temperature, resuspended in 5 mL/g pellet PBS with 0.01 mg/mL chicken egg lysozyme (Sigma) and incubated at 37 °C for 1 hour. Cell debris was cleared via centrifugation (10000 x g 4 °C, 20 min). Cleared lysate was
applied to 1 mL Strep-Tactin resin (IBA Life Sciences) and run according to the specifications. Eluted protein was concentrated in an amicon ultra centric (10 kDa cutoff) and samples were flash frozen in liquid nitrogen and sorted at -80 °C.

A.3 Results and Discussion

Induction of all constructs resulted in yellow-brown to brown pellets. The cleared lysate was also yellow-brown to brown. Over the period of an hour the color of the solution would fade when working in an aerobic environment. The induction levels varied greatly from prep to prep. The best results were attained from fresh transformations not more than two days old. Purification by Strep-Tactin resin did not work in a preparative manner. Miniscule quantities of protein adsorbed to the column even with incubation times greater than two hours.

Here we have begun an investigation of two class II hybrid cluster proteins and their associated reductases from \textit{S. oneidensis} and \textit{E. coli}. Our initial efforts have a lot of room for improvement. The loss of color during the purification process leads to the hypothesis that these enzymes are less oxygen tolerant than previously reported. The loss of color in iron-sulfur proteins often corresponds to cofactor degradation. The strep-tag does not appear to be very effective with this construct and another may prove to be more effective.

In this appendix to the larger dissertation we have described the initial attempts to study hybrid cluster proteins and reductases from \textit{E. coli} and \textit{S. oneidensis}. While our initial goal of characterizing these enzymes and investigating their electrochemical
properties has fallen short, we have effectively laid a foundation of future constructs. From here optimization of growth condition, anaerobic purification, and possibly using different expression vectors will lead to stable loaded protein to study. We have recently acquired the *E. coli* ΔiscR strain from Patrik Jones’ Lab, which has been shown to help optimize expression of H$_2$ase proteins (213). This strain has a deletion of the repressor which controls the iron-sulfur cluster maturation operon. Lacking the repressor causes the machinery to continually be produce for cluster maturation.
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Upon Interaction with Cardiolipin Bilayers .I. Evidence from Deuterium Nmr


Curriculum Vitae

Benjamin Diamon Levin

Education

Doctor of Philosophy in Chemistry  May 2013
Master of the arts in Chemistry  Awarded 2010
Boston University, Boston MA
Thesis: “Probing the structure-function relationship of heme $c$ containing bacterial proteins: mono heme cytochromes $c$ and cytochrome $c$ peroxidase”
Thesis Advisor: Professor Sean J. Elliott
- This work establishes a new understanding regarding factors that control redox potential in mono heme cytochromes and mechanistic intermediates of bacterial diheme cytochrome $c$ peroxidase.

Bachelor of Science in Chemistry  August 2003 - May 2007
University of Rochester, Rochester NY
Thesis: “EX2 Hydrogen Exchange: Mapping the Foldons of Hydrogenobacter thermophilus cytochrome $c_{552}$”
Undergraduate Advisor: Professor Kara L. Bren
- Concentration in bioinorganic chemistry/ biophysical chemistry
- Additional concentration in Music
- Worked at Carlson Library for Science and Engineering while an undergraduate.

Biochemical Research Experience

Elliott Lab, Ph.D. Candidate  August 2007 – June 2013
Boston University, Department of Chemistry
- Production of a recombinant construct for the bacterial diheme peroxidase from Nitrosomonas europaea (rNeCcP). Characterization rNeCcP using biophysical methods such as protein film voltammetry and electron paramagnetic resonance.
- Investigated peroxidase kinetics under equilibrium conditions by UV-visible spectroscopy.
- Characterized peroxidase reaction intermediates by stopped-flow spectroscopy.
- Interrogated electron transfer paths and proton coupled catalytic steps by generation of rNeCcP mutations.
- Electrochemical investigation of axial methionine loss in various bacterial mono heme cytochrome $c$.

Bren Lab, Intern and independent study  Summer 2004 – 2007
University of Rochester, River Campus, Department of Chemistry
- Studied protein dynamics and tertiary structure of cytochromes c using hydrogen-deuterium exchange monitored by 2D Nuclear Magnetic Resonance (NMR).
- Performed site directed mutagenesis to study the effects on structure, dynamics, and reduction-oxidation potential of cytochromes c.
- Investigate stability of bacterial cytochromes c utilizing circular dichroism and UV-Visual Spectroscopy.

**Doyle Lab, NSF-REU scholar**  
*Summer 2006*  
*Syracuse University, Department of Chemistry*

- Worked on expression of polyketide synthase protein actIV.
- Prepared competent cell lines for plasmid replication and protein expression using *E. coli* and *Bacillus subtilis*.
- Optimization of commercial DNA recovery kits intended for *E. coli*, for *Bacillus subtilis* and *Streptomyces coelicolor*.

**Sant Lab, Laboratory Tech**  
*May 2005-September 2005*  
*University of Rochester Medical Center, Center for Vaccine Biology and Immunology*

- Maintained T-cell lines in order to facilitate research in Major Histocompatibility Complex (MHC) Class II-Restricted Antigen Presentation.

**Publications**


**Posters and Presentations**

*Methionine ligand lability and heme ruffling in bacterial monoheme cytochromes c: an electrochemical study.*

Penn State Bioinorganic Workshop, State College, PA, June 2012.

The pH dependent behavior of axial ligation in homologous cytochromes c
ACS Undergraduate Symposium, Northeast Regional Meeting, Rochester, NY. Second place for best poster and presentation, April 2005

Teaching and Leadership Roles

Co-instructor at Penn State Bioinorganic Workshop June 2012
Penn State University, State College PA
- Co-instructor for 4 of 12 practical sessions with Dr. Elliott for practical protein film voltammetry and data analysis.
- Worked as a team to develop and test experimental modules and data analysis procedures for the practical portion of the workshop.

Bak, D., Judd, E., Levin, B., Elliott, S., Protein Film Voltammetry: SOAS Instructions and Training Modules. Penn State Bioinorganic Workshop, State College, PA, June 2012.

Organizer ChemBio Seminar Series, Fall 2008 - Fall 2011
Boston University, Boston MA
- Student run seminar series for graduate students and post docs within the chemistry department to present discuss their new work in a collegial environment.
- Secured funding each semester, arranged for space, organized schedule, and moderated the sessions.

Teaching fellow
Boston University, Boston MA
CH 421/422 Biochemistry I/II, Fall 2007 - Fall 2008
- Assisted development of new laboratory protocols and troubleshoot existing experiments under the supervision of Dr. Swapam Jain.
- Produced tutorial handout for students struggling through the laboratory report process titled “Deconstructing a Laboratory Report.”

CH 101/CH 102 General Chemistry Spring 2009 - Fall 2010
- Led discussion sections and implemented lesson plans coordinated with class and laboratory learning.
- Worked through problem sets and facilitated small group learning.
- Prepared lessons to reinforce lecture learning at heavily attended office hours.
• Performed demonstrations of general chemistry principals during lecture.

**Tutor**

*Boston University, Boston MA*  
*University of Rochester, Rochester NY*

- Provided private tutoring to students in preparation for general chemistry, organic, and biochemistry exams. Develop individual learning styles, test taking strategies, and organizational skills.