QO SITE OF THE BC1 COMPLEX; UNLOCKING THE GATE OF THE ELECTRON TRANSPORT CHAIN

BY

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DISSERTATION

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ABSTRACT

The $bc_1$ complex of the electron transport chain is critical to production of ATP through the proton gradient driven by quinone oxidation; a ubiquitous mechanism for energy conversion throughout the biosphere. In addition to being a central source of energy transduction, its bypass reactions can also lead to production toxic ROS formation from semiquinone intermediates. Its internal proton/electron shuttling and gating mechanisms have been a source of controversy and debate for decades. Structural and kinetic analysis of the $bc_1$ complex suggests several proton-coupled electron transfer processes in which gating mechanisms favor rapid forward flux while minimizing deleterious by-pass reactions. Most important are those involved in the bifurcated reaction at the $Q_o$ site. The first electron transfer normally determines the overall rate. However, control of the fate of the intermediate semiquinone involves the second step. Interplay between charged intermediates arising from the separation of proton and electron transfer pathways, and involving conserved glutamate-295 (E295), allow coulombic control, while rotation of the E295 sidechain opens a volume into which the semiquinone can diffuse towards the electron acceptor, heme $b_L$ in the low potential chain, so as to facilitate its oxidation. When E295 is mutated, these processes are so strongly perturbed that the second electron transfer becomes the rate-limiting step, as demonstrated by measurement of flash-activated kinetics of the $bc_1$ in chromatophores from wild type and E295 mutant strains of Rhodobacter sphaeroides, which show strong inhibition of $QH_2$ oxidation, and a dramatic difference in pH-dependence of activity. Attention has more recently been focused on the intermediate semiquinone, detected in the ~10-50 ms range using rapid-mix freeze-quench approaches, and shown to accumulate to low-occupancy under conditions in which its oxidation is inhibited. In our ~50ms freeze quenched studies, we showed that accumulation of $SQ_o$ in wild-type occurs only after heme $b_L$ is reduced, but that in E295W, accumulation to even higher levels occurs while the heme remains oxidized. This shows that reduction by $SQ_o$ is severely inhibited, and allows an estimate of the inhibited rate-constant. Since in this strain, the $SQ_o$ was constrained to the distal site in which it was generated, the occupancy levels likely reflect an impediment to the diffusional step that accelerates the rate constant by
shortening the distance for electron transfer. This inhibition strongly supports a mechanism in which normal forward flux requires migration of SQ₀ within the Q₀ site (2).

Until recently, study of freeze-quenched radical intermediates on the sub-millisecond time scale has not been possible. Development of microfluidic ultra-fast freeze quenching devices over the last 10 years has allowed for collection at time points as low as ~50µs. However, the expertise, resources, and time required to fabricate these microfluidic devices, coupled with their high fragility once made, have made this technique relatively inaccessible. Here we report the development of an ultra-fast freeze quenching microfluidic apparatus, using a simple lithographic construction. These microfluidic devices can be made in batches as single-use disposable components, and require little expertise, greatly increasing the accessibility of this useful technology. We have used this approach in the first experiments to monitor the formation of a SQ₀ intermediate, and establish kinetic parameters. The formation of SQ₀ was monitored in bc₁ complex from both WT and E295Q strains under different initial oxidation/reduction states by observing the paramagnetic species by EPR. Spectral analysis using CW and pulsed EPR shows a SQ₀ species not previously characterized, and has allowed resolution of some interesting structural details. These suggest a species with spin coupling both to neighboring paramagnetic states, and local nuclear magnets. Kinetic measurements show that SQ₀ is formed at a rate and occupancy consistent with a kinetic model developed to explore the diffusional hypothesis above. More detailed analysis using ESEEM suggests a strong coupling with the electron spin of the reduced 2Fe2S cluster of the iron-sulfur protein, likely involving the pyrrole nitrogen of His 152, and possibly longer-range interaction with the oxidized b-type hemes. However specific assignment of bonding/quantum coupling parameters will require more detailed spectroscopy, and quantum simulations in future collaborative efforts.
To my wonderful gracious loving and very patient wife Gloria,

To our children; Aria, Melody, and our three now in Heaven

To my parents and entire family

To Christ, Holy Mary, and all the angels and saints.

It’s been a team effort.
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**ABBREVIATIONS**

cyt cytochrome

*Cyt bc₁ complex* cytochrome *bc₁* complex

*Cyt cₜ* cyt *c₁* + cyt *c₂*

*Cyt b₁₅₀* species with *Eₘ₇* = 150 mV from interaction of SQ with heme *b₇*

DM Dodecyl Maltoside

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

ddDNA double-stranded DNA, dsDNA

dNTP deoxynucleotide triphosphate

ε-ACA ε-amino-n-caproic acid

EDTA (ethylenediamine)-tetraacetic acid

*Eₘ* mid-point potential, biochemist standard redox potential, *E°*’

EtOH ethyl alcohol, ethanol

*fbc* operon operon containing a single promoter and genes encoding the core complex (subunits ISP, cyt *b*, & cyt *c₁*)

ISPox oxidized ISP, with His-152 Nₑ of ISP is deprotonated at pH > p*K*ox₁

kcat turnover number, catalytic constant

*Kₘ* Michaelis constant, inverse of binding affinity of substrate

MOPS 3-(N-morpholino) propanesulfonic acid

PEWY highly conserved series of residues in cyt *b* subunit
pK  \(-\log K\), where \(K\) is the dissociation constant for weak acid, \(HA \rightleftharpoons H^+ + A^-\)

\(pK_{ox1}\)  \(-\log (K_{ox1})\), \(K_{ox1}\) is dissociation constant for His-152 of ISPox

\(pK_{ox2}\)  \(-\log (K_{ox2})\), \(K_{ox2}\) is dissociation constant for His-131 of ISPox

PMSF  phenylmethanesulfonyl fluoride

Q  ubihydroquinone, quinone, UQ-10 \((2\)-(isoprenyl)\)-5,6-dimethoxy-3-methyl-1,4-benzoquinone)

\(QH_2\)  ubihydroquinol, ubiquinol, quinol

\(QH\)  ubisemiquinone, neutral semiquinone

\(OH\)-decyl Q  Idebenone; \(2\)-(10-hydroxydecyl)-5,6-dimethoxy-3-methyl-1,4-benzoquinone

N-phase, P-phase aqueous phase with negative or positive proton electrochemical potential

\(Q_o\) site  catalytic site of cyt \(bc_1\) complex at which \(QH_2\) is oxidized (also \(Q_p\) site)

\(Q_i\) site  catalytic site of cyt \(bc_1\) complex at which \(Q\) is reduced (also \(Q_N\) site)

\(SQ_o\)  \(Q_o\) site SQ

\(SQ_i\)  \(Q_i\) site SQ

\(Q^-\)  ubisemiquinone anion

\(b_{H}(red)\)  reduced \(b_H\) heme

\(b_{H}(ox)\)  oxidized \(b_H\) heme

\(b_{L}(red)\)  reduced \(b_L\) heme

\(b_{L}(ox)\)  oxidized \(b_L\) heme
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SQ</td>
<td>semiquinone (dissociation state not given)</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>maximum rate, maximum velocity</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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CHAPTER 1. INTRODUCTION

1.1 Cytochrome bc\textsubscript{1} Complex and its role in the cell

The cytochrome (cyt) bc\textsubscript{1} complex (ubiquinol:cytochrome c (or c\textsubscript{2} in bacteria) oxidoreductase) and related complexes such as the b\textsubscript{6}f are central enzymes to all major pathways of the biosphere through which the redox energy of electron transfer reactions is stored in the form of an transmembrane proton gradient, and alone they account for roughly 30\% of energy transduced in this form (5). Under some metabolic conditions, they are also the site of deleterious side reactions accompanying energy transduction, leading to production of toxic reactive oxygen species (ROS) as by-products from intermediates within these enzymes. These damage both DNA and proteins, and in the free-radical theory of aging, they underlie many of the diseases of old age (1, 6). The question of how evolution has tuned the mechanism of the bc\textsubscript{1} complex so as to minimize ROS production while allowing a rapid forward flux is an important driving force for research in this area. Understanding the proper functioning of the bc\textsubscript{1} complex and its family members is of critical importance to virtually all branches of bioenergetics, from cardiomyopathy and aging to photosynthetic efficiency in agriculture, to shutting down energy production in pathogens such as malaria in drug research.

The transmembrane proton gradients produced by the bc\textsubscript{1} complex include those between the periplasmic space and cytoplasm of many bacteria. This gradient is generated via both photosynthetic and/or oxidative metabolism, by the b\textsubscript{6}f complex of the oxygenic photosynthetic chain across the chloroplast inner membrane, as well as the proton gradients across the inner membrane of mitochondria, the “power houses” of the eukaryotic cell, where the bc\textsubscript{1} complex is Complex III of the respiratory chain. The redox work available from oxidation of glucose by oxygen, or from excitation of reaction centers by sunlight, is used to generate reductant in the form of ubihydroquinone (generally UQ-10 for the bc\textsubscript{1} complex). The bc\textsubscript{1} complex (and related complexes) then catalyze oxidation of QH\textsubscript{2} by cyt c (or cyt c\textsubscript{2} in bacteria) to generate a proton gradient across the membrane. The electrochemical potential of the gradient is then available for use by the ubiquitous ATP-synthase that produces the ATP that powers the cell (1, 2, 5).
The $bc_1$ complex generates the proton gradient through the Q-cycle mechanism. The oxidation of QH$_2$ in the Q-cycle of the $bc_1$ complex is initiated in a bifurcated reaction at the Q$_o$-site, and involves two separate chains of electron acceptors. The first electron transfer is from QH$_2$ to the high potential chain (consisting of ISP, cyt $c_1$, cyt $c$, and terminal acceptor (cytochrome oxidase in the respiratory chain, or the reaction center in photosynthetic systems)), and generates an intermediate semiquinone, which is then oxidized by the low potential chain, through acceptor hemes $b_L$ and $b_H$, and the Q$_i$ site, where quinone is reduced to quinol (1, 2, 5).

This reaction-cycle involves five catalytic sites. Three of these involve external substrates; the Q$_o$-site where the incoming quinol is oxidized to quinone, and the Q$_i$ site where an incoming quinone is reduced to a quinol, and the cytochrome $c$ reductase site where heme $c_1$ is oxidized by cyt $c$ (or cyt $c_2$ in bacteria). In addition, two sites involved the extrinsic domain of ISP, one for its reduction by QH$_2$, and the other for its oxidation by heme $c_1$. Since only half of the elections from the Q$_o$ site quinol reach the Q$_i$ site, a complete turn of the Q-cycle requires oxidation of two quinols at the Q$_o$ site, to achieve reduction of one quinone at the Q$_i$ site. First, the reduced quinol enters the $bc_1$ complex Q$_o$ site, where it forms the ES-complex with ISP$_{ox}$ through which the bifurcated reaction is initiated.

When crystal structures of the $bc_1$ complex became available, the distance parameters for electron transfer reactions could be assigned, which allowed research groups to test models of the Q$_o$ site mechanism against theoretical distance-based calculations for rate constants through the Moser-Dutton equation. With recognition that the bifurcated reaction involved proton-coupled electron transfers, it was clear that while the Moser-Dutton equation was adequate for simple electron transfer reactions, an additional Bronsted term was needed to account for the properties of the first electron transfer. Structures suggested that transfer of both electron and proton must occur through the H-bond stabilizing the ES-complex, formed between the N$_e$ of His-152 of ISP (which ligands Fe2 of the 2Fe2S cluster through N$_a$) and the -OH of QH$_2$. Without the Brønsted term, the electron transfer rate constant for the ~7 Å distance would be in the range $10^8$ s$^{-1}$, or 5 orders faster than measured. If the transfer of the proton had to precede that of the electron, the rate would be slowed by the probability for a configuration in which the proton was close to N$_e$. This probability term is given by the difference in pK values of the groups par-
ticipating in the H-bond. Addition of this term to the Moser-Dutton equation gives the Marcus-Brønsted equation:

\[
\log_{10} k = 13 - \frac{\beta}{2.303} (R - 3.6) - \gamma \frac{\Delta G^{\circ}_{ET} + \lambda_{ET}}{\lambda_{ET}}^2 - (pK_{QH_2} - pK_{app})
\]

The maximal intrinsic electron transfer rate constant at van der Waals contact distance (3.6 Å) is given by the vibrational frequency of 10^{13} s\(^{-1}\), which (in log\(_{10}\) form) accounts for the value of 13. The other three terms on the right are log\(_{10}\)(probabilities) by which the maximal rate is diminished. In the first of these, the diminishment is due to the distance of electron travel in excess over van der Waals contact, with \(\beta = 1.4\) as the empirically determined slope of the plot of log\(_{10}\)k against distance, and \(R\) the edge-to-edge distance in Å. In the second term, the rate constant is reduced by a classical activation barrier, represented as in Marcus’ treatment, with \(\gamma\) set to 4.23, and \(\Delta G^{\circ}_{ET}\) (in V) as the driving force for the electron transfer step, and \(\lambda_{ET}\) as the reorganization energy (also in V). These two terms are similar to those of the Moser-Dutton equation for the electron transfer rate constant. The third term is the Brønsted log\(_{10}\)(probability) for proton configuration in the ‘active’ state, with value ~5, which knocks down the electron transfer rate constant from 10^8 s\(^{-1}\) to 10^3 s\(^{-1}\). The Marcus-Brønsted equation accounts well for the properties of the first electron transfer in wild-type, and has been tested by varying the key parameters determining the rate constant, using strains with mutations in the ISP subunit which modify both \(E_{m,pH}\) of the cluster, and \(pK_{ox1}\) of His-152. These parameters determine the driving force, \(\Delta G^{\circ}_{ET}\) and \(pK_{app}\), respectively, in the equation. With other parameters of the equation held constant, a good fit to the experimental behavior was observed for a set of five mutant strains, S154T, S154C, S154A, Y156F, and Y156W that modified the H-bonds to the cluster or immediate environment.

After the first electron is abstracted to reduce electron acceptor cyt c (which shuttles the electron to the terminal acceptor), the Q\(_o\) site quinol has now been oxidized to a semiquinone (SQ) the SQ\(_o\) intermediate, initially in the neutral form, QH\(_1\) (1, 2, 5). The SQ\(_o\) is unstable, and so acts as a powerful reducing agent, with a low enough redox potential to reduce heme \(b_L\), and drive electrons across the membrane, against the backpressure of the proton gradient to reduce the occupant (Q or SQ\(_i\)) of the Q\(_i\) site. However, the low redox potential of SQ\(_o\) means that it has the potential to react with oxygen to lead to ROS formation. This deleterious side reaction can be
prevented by rapid removal of SQ<sub>o</sub>. This is accomplished by rapid oxidation of the SQ<sub>o</sub> intermediate by the low potential chain to generate Q, which leaves the site, and is replaced by QH<sub>2</sub> to initiate a second turnover of the Q<sub>o</sub> site reaction. A second quinol oxidation at the Q<sub>o</sub> site follows the same sequence as the first, generating SQ<sub>o</sub> which is again oxidized rapidly via the low potential chain to fully reduce the Q<sub>i</sub> site quinone. As a result, one of the QH<sub>2</sub> molecules oxidized at the Q<sub>o</sub>-site is regenerated at the Q<sub>i</sub>-site, and cycled back into the Q-cycle, and the net result, as described by the reaction equation below, is that for each QH<sub>2</sub> oxidized, two protons are transferred across the membrane, and two additional protons are released into the intermembrane space (in mitochondria) or periplasm (in bacteria). See Fig. 2.

\[
QH_2 + 2cyt c^+ + 2H^+_N \rightleftharpoons Q + 2cyt c + 2H^+_P + 2H^+_{scat} \tag{1}
\]

The mechanisms behind this complex proton/electron channeling cycle have been a subject of intense study and debate for decades. One particular point of both high interest and contention has been the mechanism of electron gating between the high and low potential chains; how the bc<sub>1</sub> complex is able to channel exactly half of the electrons down each acceptor pathway even though they differ in potential by ~300mV. The two primary competing theories are (i) a concerted mechanism in which the two electron transfer reactions occur simultaneously so that no intermediate SQ species is generated, and (ii) oxidation of QH<sub>2</sub> by consecutive one-electron steps, with a transient intermediate SQ species. There are many variants of this sequential process, depending on the stability of the intermediate SQ<sub>o</sub>. With a sufficiently unstable SQ<sub>o</sub>, the reaction might appear to be concerted. An important consideration is what type of mechanism can minimize ROS production, generally considered to be the result of one-electron reduction of O<sub>2</sub> to generate superoxide as a precursor. If SQ<sub>o</sub> is the reductant for O<sub>2</sub>, then keeping its occupancy minimal would be important. However, the kinetic consequences then have to be considered, since the rate of SQ<sub>o</sub> oxidation, \( v = k_{cat}(SQ_o \text{ferriheme } b_h) \), will depend on occupancy (if \( k_{cat} \) is large enough, SQ<sub>o</sub> can be kept low). This brings up an additional issue, - that of the factors
determining $k_{\text{cat}}$, in particular the dependence on distance for electron transfer to heme $b_L$. Structures suggest that SQ$_O$ must be generated at some distance (~11.5Å) from heme $b_L$, but in a site that can reconfigure to allow a diffusional path through which it could move closer (~6.5Å) to increase $k_{\text{cat}}$ substantially (>1000-fold). This has prompted discussion of a migratory mechanism where the low occupancy and greater distance to the low potential chain is overcome by the SQ diffusing to the $b_L$ heme. The debate surrounding this question was initially theoretical, because until recently we have been unable to monitor the critical SQ intermediate. This is because under steady state conditions the high efficiency of the Q-cycle leads to the SQ existing at an extremely low occupancy. (1, 2, 3, 4). However, by blocking the Q$_i$ site with antimycin inhibitor, oxidation of heme $b_H$ through the Q$_{ii}$ site is inhibited, and the low potential chain becomes fully reduced by successive turnovers of the Q$_o$ site. Then, with nowhere to deposit its electron, the SQ intermediate builds up. Here we present the first time-resolved observations of the formation of the $bc_1$ semiquinone, obtained through our microfluidic ultra-fast freeze quenching system (2, 4, 18, 28).

1.2 Cytochrome $bc_1$ Complex in Health and Disease

The Q-cycle mechanism of the $bc_1$ complex represents a finely tuned “enzymatic ballet”, essential for proper H$^+/e^-$ shuttling and energy transduction. However, the bifurcation of the QH$_2$ oxidation reaction entails generation of SQ$_O$, which also makes the $bc_1$ complex susceptible to pathogenic short circuit reactions in which it reduces O$_2$ to superoxide, the precursor of reactive oxygen species (ROS) and other toxic radical by-products (1, 6, 32) which can be highly dangerous to the organism. The mechanism of formation of SQ$_O$, and an understanding of the conditions leading to generation of ROS by the $bc_1$ complex are obviously of importance in prevention of such damage. Certainly, the conditions under which ROS generation occurs physiologically are of high importance to cardiomyopathy and aging. Our studies of SQ$_O$ formation under different redox conditions are in particular pertinent to functional recovery after ischemia in both heart attack and stroke. Our demonstration of increased occupancy of SQ$_O$ on delivery of oxidized cyt c to the partially or fully reduced $bc_1$ complex provides a direct insight into why damage occurs on oxygenation after ischemia. In the presence of O$_2$, cytochrome oxidase will generate oxidized
cyt c, and the SQ\textsubscript{0} generated by the above mechanism will be consumed by generation of superoxide, and consequently of ROS. These mechanisms offer potential drug targets (7, 8, 9).

1.2.1 Mitochondrial and aging. While the direct cause or causes of aging continue to be debated with numerous theories (10), the free radical theory of aging remains prominent. Whether it be from ROS-induced damage to mitochondrial DNA, or from ROS acting as a signaling molecule, or both, the role of the ROS species in aging is clear, and the \( bc_1 \) complex is a major source of ROS production (10, 32). As shown in figure 1.1, there are a large number of mutations centered around the Q\textsubscript{0} site of cyt \( b \), here shown modeled on the \textit{Bos taurus} cyt \( b \) structure, that are linked to mitochondrial and cardiomyopathies as well as accelerated aging. The critical nature of the Q\textsubscript{0} site region is highlighted by the fact that all documented mutations are on the periphery and not within the Q\textsubscript{0} site itself. While a peripheral mutation partially damaging the Q\textsubscript{0} site may lead to various documented myopathies, an internal mutation shutting down the Q\textsubscript{0} site would result in very premature embryonic death.

1.2.2 Malaria. Malaria is one of the most devastating diseases in the third world where its mortality rate among infected individuals is enhanced by a lack of infrastructure, nutrition, and sanitation. Most tragically, the WHO reports that 90% of those killed in 2013 by malaria were children under five in Africa; over half a million children (11). While great strides have been made recently in prevention and control of this widespread disease, and it has been largely eliminated from much of the global population, its continued resistance to current drugs gives it the potential to return to pandemic levels globally, with 3.3 billion people currently at risk of infection, and up to 283 million cases every year (11).

One of the most promising recent drug candidates against malaria, atovaquone, has been found to specifically target the \textit{P. falciparum} \( bc_1 \) complex Q\textsubscript{0} site. However, while atovaquone is still an effective drug, resistance to atovaquone was developed by \textit{P. falciparum} very early on (7). Detailed knowledge of the Q\textsubscript{0} site structure-function relationships are critical to develop more effective drugs against the \( bc_1 \); both in malaria and other pathogens.

The continued effectiveness of atovaquone despite widespread resistance by the malaria parasite, is through the fact that strains of \textit{P. falciparum} that have developed resistance often accom-
plish this only at the expense of virulence. In one example, the Y268S cyt b mutation leading to atovaquone resistance comes with a 40% reduction in $V_{\text{max}}$ (12, 13) in the damaged $bc_1$ complex. The parasite is able to compensate with increased expression in this mutant, however resistance being coupled with decreased parasite fitness is common (13).

1.3 Cytochrome $bc_1$ Complex: Structure and Function

Although the number of subunits within the $bc_1$ complex and their sequences can vary significantly between the branches of the tree of life and between species (14, 5, 32), the catalytic core is contained in three highly conserved subunits, so that the $bc_1$ complex shows high mechanistic and structural conservation across the biosphere (32) as evidenced in crystal structures (14), in high conservation of key residues within the active site (14), and conservation of the Q-cycle mechanism.

The $bc_1$ complex used in our studies is from the photosynthetic bacterium *Rhodobacter sphaeroides*. Despite the ~2 billion years of separation between genetic pools, it functions using the same mechanism as the mitochondrial $bc_1$ complex. *Rb. sphaeroides* has been chosen for our studies for several reasons; i) there exists an established and well-tested purification protocol for the isolated complex that facilitates *in vitro* studies, ii) the bacterial location makes it easier to produce point mutations, iii) the location in photosynthetic electron transfer chain allows photo-activation through the reaction center, allowing measurement of kinetic in the sub-ms range; iv) the bacterial membrane invaginations containing the photosynthetic apparatus generate chromatophore vesicles on disruption, to allow for facile kinetic studies of a self-contained apparatus, and v), the *Rb. sphaeroides* $bc_1$ complex is simpler than the mitochondrial complexes because it contains only four subunits, the catalytic core plus a minor subunit IV of uncertain function.

All $bc_1$ complexes are homodimeric, with a core of three subunits in each monomer; cytochrome $b$ (cyt $b$), the ISP, and cyt $c_1$. *Rb. sphaeroides* also co-purifies with a fourth subunit, “subunit IV” (SU IV) of unknown function and structure (5, 14). While many of the 11 mitochondrial subunits also have unknown function, the conserved core subunits have a high degree of sequence homology with the $\alpha$-proteobacterial $bc_1$ complex core. In other major branches of the family, for example the $b_{6f}$ complex of green plants and cyanobacteria, the ISP and cyt $b$ subunits are largely conserved, but the heme $c$ group is contained in a structurally unrelated cyt $f$. 
not within a cyt c1 (25), and, in firmicutes, in a cyt c subunit which can also be unrelated to cyt c1.

In addition to bc1 complex crystal structures from a broad range of organisms across the tree of life (Animalia, Plantae, Fungi, and Prokarya), a much wider range of organisms have been studied through sequence homology, and the high degree of conservation in the catalytic core suggests a highly conserved Q-cycle mechanism.

1.4. The second electron transfer of the bifurcated reaction

Detailed analysis of kinetic and thermodynamic properties of the bc1 complex in wild-type and strains with mutations at key residues has allowed definition and quantification of physiochemical parameters for many partial processes of the Q-cycle as described below. The bifurcated reaction at the Qo site is the focus of our work, and can be summarized with five primary points (19, 29, 30, 31, 33); figure 2.

1. Since the ISP extrinsic domain is mobile between the cyt c1 and cyt b interfaces, there are effectively two substrates involved in the formation of the ES-complex; the incoming QH2 and the ISPox domain itself. In the ES-complex, His-152 (H152) of ISPox, a ligand to the Fe2S2 cluster, forms an H-bond with QH2 as suggested by stigmatellin-occupied crystal structures (37).

2. With saturating QH2, the proton-coupled electron transfer to ISPox through H152 represents the rate limiting step of the Q-cycle, with the ISP mobility being rapid enough to not have a significant affect. The ES-complex with its two substrates is readily formed, but only under metastable conditions. It is fully populated at Eh,7 ~90 mV, where the quinone pool is half reduced, when turnover of the reaction center generates excess ferricyt c2 to oxidize the high potential chain. The first electron transfer at ~1000 e-/cyt b/s is rate limiting, and the second occurs at high enough rate under normal conditions to keep the SQo occupancy at undetectable levels. The properties of the first electron transfer suggest that the SQo is unstable and reaction is strongly endoergic, so that it is pulled over only because the second step is sufficiently exoergic. Although the SQo is generated in the first electron transfer, its fate is determined by the second electron transfer.
3. Following the first proton/electron transfer to the ISP\textsubscript{ox}, generating ISPH with the concordant QH\textsuperscript{-} neutral SQ formation, a second and more controversial proton/electron transfer occurs. In this second proton/electron transfer, the electron is transferred to heme b\textsubscript{L}. However, the proton is shuttled out through a separate path likely involving Glu-295 (E295) of cyt b, and a water chain. This second proton/electron transfer determines the fate of SQ\textsubscript{ox} and its observable kinetics, which is the subject of this work. The product Q has significantly lower affinity for the Q\textsubscript{O} site than QH\textsubscript{2}, encouraging dissociation.

4. The SQ\textsubscript{O} product of the first step, whose existence as an intermediate species was, until recently, controversial, is relatively unstable.

5. The oxidation of the unstable SQ intermediate is exergonic, and occurs rapidly as long as ferriheme b\textsubscript{L} is available to act as acceptor. This pulls the reaction forward thermodynamically. As a consequence of this rapid second proton/electron transfer, the reaction appears to be concerted under these conditions, and the SQ\textsubscript{O} intermediate occupancy levels are too small to be detectable at steady state in the wild type bc\textsubscript{1}. (Fig. 4)

\[
\begin{align*}
&\text{Eb}_{\text{L}}, \text{QH}_2 \\
&\text{QH}_2 \rightarrow \text{ISPH}^\text{ox} \\
&\text{Eb}_{\text{L}, \text{QH}_2, \text{ISP}^\text{ox}} \rightarrow \{\text{ES}\}^x \rightarrow \text{Eb}_{\text{L}, \text{QH}}^+ \rightarrow \text{HISP}^\text{red} \rightarrow \text{Eb}_{\text{L}, \text{QH}}^+ + \text{Q} + \text{HISP}^\text{red} + \text{H}^+
\end{align*}
\]

\textit{from (33)}

While the very low levels of SQ\textsubscript{O} complicate investigation of its proton/electron transfer mechanism, it is highly advantageous for the cell as it minimizes the potential for reaction to form toxic ROS. Under circumstances in which the electron transport chain has become reduced by O\textsubscript{2} shortage due to a restriction in blood flow (ischemia), it has been suggested that re-oxygenation can lead to a buildup of SQ\textsubscript{O}. Damage to heart or brain tissue under such circumstances has been recognized in the last decade, leading to modification of recommended medical practice so as to avoid use of O\textsubscript{2} to increase oxygenation levels. The accumulation of SQ\textsubscript{O} under
similar metabolic condition has been demonstrated \textit{in vitro} in this work, validating for the first time the mechanism justifying this change in practice.

1.5 Mechanism of Quinol Oxidation at the Q_0-site; Controversy

The lack of data on the SQ_0 has led to the proposal of widely differing mechanisms in the recent literature to explain the second proton/electron transfer. These mechanisms have included a Q_0-site model of double quinone occupancy, concerted first and second proton/electron transfers, and diffusion of the SQ intermediate within the Q_0 site.

The quinol oxidation/reduction enzymes, cytochrome \textit{bo}_3 (34) and the photosynthetic reaction center (36), both use stationary tightly-bound Q cofactors for their electron transfer mechanisms, and given the size of the Q_0 active site, it seemed natural to speculate on a similar double-occupancy model with two Q_0-site quinols (35). However, the lack of any tightly bound Q_0 site quinone in any of the crystal structures cast significant doubt on this hypothesis over a decade ago.

Before Cape et al. (4) demonstrated SQ_0 formation, a concerted mechanism was widely favored, largely due to the failure to detect any SQ_0 sensitive to Q_0 site inhibitors. No visible SQ_0 would be expected under the concerted model, where both proton/electron transfers would occur simultaneously from the distal domain of the Q_0 site. However, interpretation of crystal structures had already cast doubt on this theory before Cape et al. (4). Initially, application of the Moser-Dutton equation to Q_0-site distances showed that the observed rates, though technically consistent with theoretical rates of a concerted model, would require much higher occupancies than seemed feasible for such a model (see below).

As structures became available of the bc_1 co-crystallized with quinol analogs bound to the Q_0 site, a paradoxical feature relating to the efficiency of bifurcation was noted. If the \textit{ES}-complex forms in the configuration involving H152 of ISP suggested by the structures, then the formation of SQ_0 would occur in the same position. Then, once ISP_{ox} returned from oxidation by heme \textit{c}_1, the SQ would be far closer to ISC, as a competing acceptor, than to the \textit{b}_\textit{L} heme. Since ISP has a
much higher $E_m$, both distance and driving force would favor transfer of the second electron to the cluster, rather than the heme, raising the question of what could allow the second proton/electron transfer to be directed to heme $b_L$. Several suggestions have been discussed involving gating mechanisms in both acceptor chains. In the present context, the possibility that SQ$_O$ might diffuse in the site to decrease the electron transfer distance, and so enhance the rate constant with heme $b_L$ has received most attention. The first clues to a possible diffusional model came from differential binding of varying inhibitor types to differing regions of the Q$_O$ site; the “distal” domain ~7Å from the ISC and ~12Å from heme $b_L$, and the “proximal” domain ~7Å from the heme $b_L$ and ~12Å from the ISC. As described in (32), since stigmatellin was found H-bonding with the ISP, the inhibitor was bound in the domain distal from heme $b_L$; however, myxothiazol and MOA-type inhibitors occupied the domain proximal to the heme. In the stigmatellin structure, the proximal domain was occupied by the sidechain of the conserved PEWY glutamate (E295). Of particular interest, the locations of myxothiazol and MOA-type inhibitors in these domains was associated with the ~130° rotation of E295 sidechain. In effect, rotation of the sidechain opened up the proximal volume so that myxothiazol could fit. It was therefore suggested that a similar rotation might allow the SQ$_O$ anion to migrate towards heme $b_L$; the change in distance would be expected to increase the rate constant for the second electron transfer by 1000-fold. In later developments, controlling and gating between high and low potential chains has been discussed in the context of coulombic interplay resulting from separation of H$^+$ and electron also involving E295, as a type of biochemical metronome (See Fig. 3).

Kinetics of Q$_O$-site point mutants at PEWY glutamate E295, which in a simple Moser-Dutton treatment would not affect electron transfer, showed a very significant inhibitory impact, strongly suggesting a more complex process, in line with the role proposed in the diffusional SQ$_O$ model (1, 2). Briefly, E295 mutants switched the rate limiting step of the $bc_1$ complex from the first proton/electron transfer to the second, while lowering the overall $V_{\text{max}}$ by a factor of >50, depending on pH. The $bc_1$ turnover rates could be successfully plotted against a model of the Q$_O$ site reaction including the diffusion-based second proton/electron transfer where the effects of mutation were simulated by decreasing the rate of movement within the Q$_O$ site from an effective rate constant of $\sim10^7$ to $\sim10^2$ s$^{-1}$ for the diffusional step.
As noted above, a key source of the controversy surrounding the $Q_o$ site mechanism has been the difficulty in observing the $SQ_o$ intermediate. However, as shown by Cape et al. (4), by blocking the low potential heme electron release by adding the $Q_I$ site inhibitor antimycin and taking a sample at a time when at least two $Q_o$ site turnovers could have reduced both $b$-type hemes, since no acceptor is available, the $SQ_o$ species accumulates. In E295W, the especially slow rate of electron transfer allows the $SQ_o$ to accumulate under conditions in which heme $b_L$ is mainly in the oxidized form. With the bulky W residue acting as a source of steric hindrance, the $SQ_o$ is most likely confined to the distal domain (see Fig. 5), allowing determination of both $[SQ_o]$, $[ferriheme b_L]$, and a rate of electron transfer (2). From this information, the intrinsic rate of the second proton/electron transfer to the $b_L$ heme can be directly calculate, giving a value of 1000 s$^{-1}$, which is three orders of magnitude smaller than the rate expected for electron transfer from the distal domain distance suggested by structures. The latter value would have yielded the observed rate only at $SQ_o$ occupancies at the high end of those expect from kinetic measurements of lag-phases. Our results with E295W study (1, 2, and see Chapter 3) are consistent with the diffusional model, but the empirical value excluded models in which electron transfer occurred from the distal domain. In particular, since concerted mechanisms require occupancies in the concentration range of the activation barrier, such models were definitively excluded.

1.6 Previous $Q_o$ site SQ Studies

Zhu et al 2007. A study was previously conducted by Zhu et al. (3) to test the hypothesis that the bifurcated mechanism proceeded through a SQ intermediate, by monitoring kinetics of paramagnetic species ferriheme $b_L$, ISPH and $SQ_o$ through ultra-fast microfluidic freeze-quenched EPR. They used high $bc_1$ concentrations that allowed them to observe oxidized hemes $b_L$, $b_H$ and $c_1$ and ISPH with relatively high sensitivity. In particular, by omitting ferricyt $c$ as acceptor, they avoided interference by signals from the excess concentration needed for acceptor function. In this technical tour de force, they determined the early kinetics of the high and low
potential chain components in the uninhibited complex over the range 66 μs to 3.0 ms, and demonstrated that electron transfer to the two acceptors, heme $b_L$ and ISC reached a level of ~10% reduction within 400μs, apparently with identical kinetics, and without formation of SQ$_o$ within the detection limit of their experiment. SQ detected in the ms range was attributed to SQ$_i$ formation. They conclude that no SQ intermediate was involved in the bifurcated reaction. However, our detailed analysis in the context of a realistic kinetic model incorporating the diffusional step (1, 2) showed that under conditions in which ferriheme $b_L$ was available as acceptor, the occupancy of SQ would be well below their detection limit. Since their data were obtained using the uninhibited complex, and since the Q$_o$-site reaction is rate limiting, no visible SQ$_o$ would be expected to accumulate, because any reduction of heme $b_L$ would be followed by rapid re-oxidation though turnover of heme $b_H$ and the Q$_i$ site.

Under a single turnover reaction, our SQ Q$_o$ site diffusion model expects a result similar to their own, given their experimental methodologies. Kinetic simulations predict occupancy of SQ$_o$ intermediate on the order of <0.0001% for a single turnover reaction for the diffusional SQ model. This occupancy would be far too small to be detectable by CW EPR, even with their high $bc_1$ concentration. The Zhu et al. study provides a useful validation of our model under the conditions of their experiment, but certainly does not exclude mechanisms involving a SQ intermediate in the bifurcated reaction.

**Cape et al. 2007.** In this study, rapid-mix freeze quenching was conducted *in vitro* on initially oxidized $bc_1$ complex inhibited with antimycin but without added acceptor, and reacted with chemically reduced decyl-UQH$_2$. Impressively, the freeze-quench was initiated at10ms through the use of liquid propane as the cryobath medium. This was the first study where the Q$_o$ site SQ was clearly observed. They show 0.01 to 0.1 SQ occupancy per monomer. From the well-characterized thermodynamic and kinetic properties of the Q$_o$ site reaction, this high occupancy was somewhat surprising, because no accumulation would have been expected until heme $b_L$ became reduced, but this would have required two turnovers of the Q$_o$ site, which would have consumed both acceptors (ISC and heme $c_1$). Then, the driving force would have disappeared for
generation of SQ₀.

It is conceivable that some residual oxidizing species was present from preliminary oxidation of the complex, and available to drive the multiple turnovers needed to reach the occupied condition observed. Alternatively, since the reaction was performed anaerobically, their initial conditions may have allowed pre-reduction of heme b₄₅ so that only one turnover would be needed before SQ accumulated. This might also account the high sensitivity of SQ accumulation to oxygen reported. The fact that the occupancy levels from our own data, obtained under conditions similar to theirs, were similar but only when excess cytochrome was added as acceptor (1,2,4), is in line with expected properties, but suggests some unacknowledged artifact in their methodology.

**Zhang et al. 2007.** In this report, formation of SQ at the Q₀ site was detected in chromatophores after illumination to generate the substrates *in vivo* through photoactivation of the reaction center. Occupancies reported were much lower than in the Cape et al. work, likely, as discussed elsewhere (1, 2), because the sample was collected after the P⁺ signal had decayed to undetectable levels, indicating that the driving force from oxidants in the high-potential chain was insufficient to maintain higher occupancy.

**Sarewicz et al. 2013.** In this impressive study, the Q₀ site SQ was once again generated through mix-hold cryobath freeze quenching, with maximal occupancies in the range of ~20%; similar to the 10% reported by Cape et al 2007 (4). Sarewicz et al. (26) is the first publication to report evidence of a binding interaction with the SQ through the ISC, albeit on a non-physiological time scale of seconds to minutes.

The kinetics of the formation of the SQ-ISC complex in (26) were observed over several seconds to minutes; their shortest time point was at 12ms, and the complex reached maximal occupancy at ~12 s, and then declined to zero at 2 min. These kinetics are thousands of times slower than needed for normal turnover. Since their bc₁ complex samples were pre-incubating with 8-fold excess of ferricytochrome c as acceptor, followed by mixing with 12-fold excess of decyl-UQH₂, multiple turnovers of the Q₀-site might have been expected. In the absence of antimycin, the 2Fe2S cluster remained oxidized during the first 500 ms, and reached full ½ reduction at ~1s;
this time course was lengthened in the presences of antimycin, reaching ½ reduction at ~ 10s, approximately when the SQ-ISC signal reached maximal amplitude. However, unexpectedly, no heme $b_L$ reduction was observed even several seconds after mixing in samples inhibited by antimycin, with or without myxothiazol. The high level of oxidation shown by the ferriheme $b_L$ signal leads them to their hypothesis that the reaction proceeds in reverse, with a high SQ population generated in equilibrium with heme $b_L$, but could involve either reduction of Q and equilibration with the Q/SQ couple ($E_{m} < -300 \text{mV}$), or reduction of SQ$_o$, and equilibration with SQ/QH$_2$ couple ($E_{m} > \sim 500 \text{ mV}$). However, the former scenario would be in contradiction with the observation of ferriheme $b_L$, and the low redox potential of the Q/SQ compared to the -90mV redox potential of the $b_L$ heme as donor (see Fig. 1.4).

While the kinetics and mechanisms by which the observed SQ-ISC coupled species is formed in their study are certainly open for discussion, the observation of this coupled species is significant. Until recently, the formation of a SQ-ISC coupled species as a possible means of preventing bypass reactions had been only a matter of speculation (6).

**1.7 Freeze Quenching**

Until recently, the standard methodology for freeze-quenching has been an apparatus wherein reactants are rapidly mixed, and then allowed to incubate for an adjustable amount of “hold” time, before ejection into a bath of organic solvent, a “cryobath”, which has been brought down to approximately -150°C by liquid nitrogen. The time scale of the reaction can also be varied by adjusting the distance from the mixer to the cryobath. Such a system has been available commercially for some time, and was used by Cape et al. (4) and by Victoria et al. (2) to generate the Q$_o$ site SQ at 10ms and 50ms, respectively.

For time resolved studies of EPR signal, the only methodologies available are chemical quenching and freeze quenching of a reaction, with freeze quenching being far less invasive. The fundamental drawback of currently commercially available freeze-quenching equipment is that they are limited in time scale to a minimum of ~10ms. For many enzymes this time scale is useful, but for the $bc_1$ complex, with a turnover time ~1 ms, a better kinetic resolution down to the sub-ms range is need if reaction mechanism is to be explored. In recent years the emerging field of microfluidics has allowed for freeze quenching time points as low as 66µs (3, 37). However,
due to the technological expertise and resource-intensive nature of the microfluidic technique used, and the high fragility and expense of the device, this technique has been inaccessible to most labs. In this work we present the development of a facile methodology for jet-forming microfluidic mixer fabrication as single-use disposables at insignificant cost.

The use of microfluidics is transforming the field of freeze quench kinetics largely through the simple fact that the kinetics of heat transfer is inversely proportional to the thickness of a material. A jet of solution with a diameter of 20µm will freeze at least 25 times faster than a jet of solution with a diameter of 500µm (see Fig. 1.6). However, there many other factors come into play as well:

i) In the mix-hold approach, a jet of relatively large diameter containing a large volume is injected into a cryobath over a matter of milliseconds, and a kinetic point is determined by a sum of mix time, hold time (limited by the rate of ejection valve switching), and time-of-flight. In contrast, in micro-fluidic approach, a jet of much lower diameter, sustained over several seconds to minutes, with a flow rate of only 8 µL per second, is splattered on a spinning copper wheel maintained at liq. N\textsubscript{2} temperature, and the time of reaction beyond the mixing time and minimal flow distance (~30 µs) is determined almost entirely by time of flight. With the flow rate used, the reaction time can therefore be finely tuned with an accuracy dependent on control of distance over the range 1.0 mm to 10 cm.

ii) Since the flow rate of the micro-fluidic jet (8 µL.s\textsuperscript{-1}) delivers a relatively small volume per unit time, the jet can be directed onto a metallic surface, - a copper wheel cooled directly by immersion in liq. N\textsubscript{2}, - without the sample building up on top of itself to slow the freezing. In our configuration, the surface is renewed by contact with a counter-revolving wheel so that the sample at the contact point is pulverized, and released to the liq. N\textsubscript{2} bath.

iii) A freezing surface with a metal of high thermal conductivity can be maintained at true liquid nitrogen temperature, while the cryobath medium must be kept above -150\degree C or else it solidifies.

iv) The cryobath solutions such as the liquid propane used in (4) or the pentane used in (2) are highly flammable, and potential explosive, requiring special safety measures.
v) The freezing process when a liquid jet is sprayed on, or injected, into a cryobath liquid introduces problems in assigning a time for the kinetic sample. Several additional delays are introduced by the time for freezing, and by heterogeneity in this time associated with the volume involved: the frictional lag limits the volume of cryo-liquid seen by the sample; clumping of the sample increases the volume; the freezing time varies depending on the heat flow from the surface to the interior of the droplets. Heterogeneity introduced by this latter effect can stretch the time of freezing over tens to hundreds of ms. However, when a liquid jet hits the solid revolving freezing surface, it flattens out, and this has the effect of accelerating rather than slowing freezing, thus reducing heterogeneity in the sample.

The ultra-fast mix-flow-freeze quenching method introduced here allows us to collect samples for EPR measurement on the time scale of 166µs to 4ms. We can then assay relative concentration of paramagnetic species by CW EPR to determine the early kinetics of the Q_o site reaction. The same samples can be used for direct studies of the local environment of such species by pulsed EPR detection and characterization of nuclear spins in the immediate reaction volume. This has been accomplished in this study across multiple redox states of the WT bc_1 and the E295Q mutant.

**Freeze Quenching Technique and Spectrum Shape.** As demonstrated by Georgieva et al. (28), ultra-fast freeze quenching on the microsecond time scale is able to capture a broader range of high-energy conformers than are obtainable with slower freeze quenching methods such as direct insertion into liquid nitrogen. The freeze quenching methods employed so far in the literature that successfully observe the Q_o site semiquinone have employed ejection of bc_1 complex into a cryobath with an average freezing time of at least 10ms. However, the freezing time used in our studies is roughly three orders of magnitude faster, meaning less time for frozen samples to relax to lower energy states in their new landscape, and a far more reliable conformation distribution becomes visible.

In addition to solute having less time to change conformations to adapt to a frozen solution energy landscape, the ultra-fast freezing method can also change the frozen solution energy landscape itself. Freezing the solution before a high degree of crystallization and solute segregation
can occur as discussed in Ross et al. (27) can lead to the sample forming an amorphous ice, while slower freezing methods result in a less physiological crystalline ice Georgieva et al. (28). This slow-forming crystalline ice can lead to solute segregation and solvent/solute phase separation; forcing groups into non-native conformations and heterogeneous high local concentrations. Heterogeneous high local concentrations from solute phase separation can result in dipolar line broadening due to interaction with adjacent paramagnetic solute (27). This factor can have significant impact on the EPR spectrum (27), but is largely ignored in the literature. The addition of additives such as glycerol will significantly mitigate, though not eliminate, this affect.

In the Georgieva et al. (28) study, Double Electron Electron Resonance (DEER) was employed in site-directed spin labeling to produce double-labeled T4 lysozyme. T4 lysozyme is a very well characterized protein with a relatively rigid structure, making it a good choice to study the effects of freezing times on frozen conformers. Through DEER, distance between spin labels measured in nm, and P(r), the relative probability of a given distance between labels, were plotted. As expected, no change was observed in the average distance between spin labels, however a significant change was observed in the distribution of populations of spin label rotamers/conformers across the average. Naturally, the more degrees of freedom and differences in available energy states, then the more of an impact a shift in energy state populations may have.

A similar affect to the Georgieva et al. and Ross studies (28, 27) are observed in our study on bø3 where identical samples of bø3 were frozen by either ultra-rapid freezing, or slowly frozen by direct insertion of EPR tubes containing bø3 into liquid nitrogen (data not shown). Analysis by CW X-band EPR shows that the number of peaks and approximate intensities observed between the two freezing methods remains the same, however, the peaks are far less defined in the slowly frozen sample, where to a large extent they merge into one central peak.
1.8 Electron Paramagnetic Resonance

1. Continuous Wave EPR

Electron Paramagnetic Resonance, or EPR is a technique analogous to NMR, however in the microwave frequency range appropriate to electron spin, for example of ~9GHz for X-band and ~35 GHz for Q-band, in contrast with radiowave frequencies appropriate to the nuclear spins of NMR. The large frequency range available for EPR studies (S-, X-, Q, W-bands are available commercially) allows for the analysis of paramagnetic species with a wide range of properties, and makes EPR ideal for monitoring and characterizing any different free radical reaction intermediates.

In continuous wave (CW) EPR, the sample with paramagnetic species is irradiated with microwaves of a fixed frequency, and the magnetic field is varied to search for microwave absorption at resonance with the sample. Looking for the dependence of microwave absorption on the magnetic field as described in the equation below for a simple uncoupled system:

\[
hv_o = g_e \beta_e B_o
\]

\[
N_+/N_- = \exp(\frac{\Delta E}{kT})
\]

Where \( h \) is Planck’s constant, \( g_e \) is the dimensionless electron g-factor, \( \beta_e \) is the Bohr magneton constant, and \( v_o \) and \( B_o \) are the resonant frequency and magnetic field at resonance, respectively. \( N_+/N_- \) is the ratio of the (+) and (-) spin states, \( \Delta E \) is the energy difference between these populations, \( k \) is the Boltzmann constant, and \( T \) is the temperature of the experiment. The magnetic field with resonant frequency \( v_o \) induces shifts from \( N_+ \) to \( N_- \) and vice versa with equal probability, however the transition is naturally weighted towards \( N_+ \) since the initial population is weighted towards \( N_- \), leading to the ability to overall absorption of microwave photons.

\[
hv = \Delta E
\]

As the magnetic field is increased, the (+) and (-) spin states become more separated as the magnetic field pushes them further apart. This alters the \( \Delta E \) between the two spin states. When the \( \Delta E \) between the (+) and (-) spin states matches \( hv \), where \( v \) is the fixed frequency of incoming microwaves, then microwave absorption, or ‘resonance’ occurs.
II. Pulsed EPR

Pulsed EPR, which is analogous to pulsed NMR, allows for direct structural data to be collected on the nuclear environment of paramagnetic species within a sample, making it ideally suited to determining the structure/function relationships behind mechanisms involving radical intermediates.

In CW EPR, as discussed above, the magnetic field changes with the microwave frequency is held fixed. The same principle applies in pulsed EPR, but instead of a continuous microwave excitation, the sample is treated with pulse of microwave excitation at the magnetic field strength appropriate for resonance at some point of interest in the CW spectrum (39). The microwave pulse flips the spins of the excited population, and the duration and sequence of these pulses determines the change in vector of spin alignment for the paramagnetic species. Commonly pulse duration is chosen so as rotate the vector by 90° (a $\pi/2$ pulse) or by 180° (a $\pi$ pulse). After flipping, the spin population begins to defocus/decay back to ground state orientations. The process of this relaxation leads to the generation of a transient microwave signal (the spin echo) whose frequency and decay time vary depending on the properties of the species (39). Detection of the spin echo is the basis of all signal collection in pulsed EPR.

**Electron Spin Echo.** This is one of the simplest varieties of pulsed EPR, and is usually run before a more complex pulse sequence to verify the quality and g-value of the sample. Initially, sample oriented along the z-axis is excited with a $\pi/2$ pulse, which orients the magnetization vectors along the y-axis, after which the spin population begins to defocus over the x-y plane. By then hitting the defocusing magnetization vectors with a $\pi$ pulse, their vectors are rotated 180°, causing them to reverse direction and go back to aligning at the y-axis, which they all reach at the same time. This refocusing effect produces a microwave emission called the two-pulse electron-spin echo (ESE) (39).

When such an experiment is conducted with the field varied through the range of resonance for a particular species, the amplitude of the spin-echo varies, and the result is a field-sweep spectrum, which is, in effect, related to the integrated CW spectrum. However, by varying the properties of the pulse sequence, different features of the paramagnetic species, for example
the decay-time of spin state, can be explored. The field swept ESE spectrum is therefore a useful tool, though not as sensitive as CW EPR, which is normally used for higher quality data.

**Electron Spin Echo Envelope Modulation.** This approach might involve many different variants of pulse sequence, and can give direct structural information about the environment of a paramagnetic species through analysis of the “echo envelope”. The echo envelope is obtained through the intensity of the echo signal as a function of the timing between pulses, which maps out the perturbation of the relaxation kinetics by interaction with neighboring nuclear spins. Fourier analysis of the time course reveals the frequency of these interactions, which then can be interpreted in terms of the atomic identity, interaction energy, angle of H-bond, etc., thus allowing for a mapping of the local nuclear environment contributed by protein and solvent.
Fig. 1.1 Mutations in cyt $b$ have been directly linked with multiple cardiomyopathies. Upon folding of cyt $b$ (*Bos Taurus* structure shown; 79% identity, 89% similarity to human) there is clear clustering of the clinically relevant mutations around the $Q_0$ site. Stigmatellin $Q_0$ site inhibitor is in yellow.
**Fig. 1.2 The Q cycle** Flow of electrons shown as blue arrows for one full Q cycle with two turnovers of the \(Q_o\) site and one turnover of the \(Q_i\) site.
Fig. 1.3 Migratory model of the second $Q_0$ site electron transfer.

**A)** High potential chain ($1^{st}$ rate limiting $e^-$ transfer; $\sim$7 Å)

$$E_{b_H}b_L.QH_2.ISP_{ox} \rightleftharpoons ISP + E_{b_H}b_L.QH$$

$$ISP + \text{heme } e^+ \rightleftharpoons ISP_{ox} + \text{ferroheme } c_1 + H_2^+$$

**B)** Low potential chain ($2^{nd}$ $e^-$ transfer; $\sim$12.4 Å distal, $\sim$6.3 Å proximal)

$$E_{b_H}b_L.QH \rightleftharpoons E_{b_H}b_L^- + Q + H_2^+$$

$$E_{b_H}b_L^- \rightleftharpoons E_{b_H}b_L$$
Fig. 1.4 Free energy diagram of the Q cycle with reaction coordinates.
Fig. 1.5 Theoretical inhibitor-based structure of QH$_2$ bound within the distal region of the Q$_o$ site. Highlighted residues are E295; the theorized “gate” for SQ$_o$ migration, Y147, the theorized proton acceptor for channeling to the water chain, as well as H156; participating in proton transfer as well as electron shuttling between the QH$_2$ and the Fe$_2$S$_2$

Fig. 1.6 Freeze quenching time increases with the cross sectional diameter of the jet.
Fig. 1.7 Basic continuous wave energy splitting
CHAPTER II. EXPERIMENTAL PROCEDURES

2.1 Materials

MOPS, KCl, MgCl$_2$, PMSF, $\varepsilon$-ACA, and antimycin were from Sigma. Biochemical reagents were obtained from commercial sources. Horse heart cytochrome $c$, antimycin-A, and decyl-ubiquinone, were purchased from Sigma and used without further purification. POPC was obtained from Avanti-biolipids. Ascochlorin was a kind gift from Dr. Nobuko Minagawa, Department of Biochemistry, Niigata University of Pharmacy and Applied Life Sciences, Niigata 956-8603, Japan. Purified NDH-2 enzyme from *E. coli* was a kind gift from the lab of Robert Gennis. SU 8-25 photoresist, silicon wafers, PDMS resin, and PTFE tubing were all kind gifts of the Kenis lab, as was the use of their microfluidic fabrication facilities.

WT and mutant *R. sphaeroides* strains were developed in Crofts lab as described in (2, 44) where the genomic operon for $bc_1$ expression was replaced in the chromosome by a kanamycin cassette, and inserted into a plasmid for facile generation of point mutations, and also for engineering to add a His-tag to cyt $b$ to facilitate purification through use of an affinity column.

2.2 Methods

2.2.1 *R. Sphaeroides* cell growth. *R. sphaeroides* cells were grown photosynthetically as previously described (2). Cells cultures were progressively scaled up in volume from 3ml to 40ml aerobic growth, to photosynthetic growth in 1L cultures for 3 to 5 days, followed by photosynthetic growth in 2 to 4 5L cultures until cell growth was in log phase. In the case of $bc_1$-defective mutant strains E295Q and E295W, 0.05% DMSO was added to sistrum media to allow for growth during the photosynthetic stages. All cell preps were in the presence of tetracycline (2 $\mu$g/mL) and kanamycin (20 $\mu$g/mL).

For all cultures of E295 mutant strains, any possible reversion to wild type was monitored through DNA sequencing prior to protein purification from harvested cells. Plasmid from harvested cells was isolated using a Qiagen Miniprep kit, and sequenced at the UIUC Core Sequencing Facility following PCR amplification of the E295 region of cytochrome $b$. Mutant reversion to WT was also monitored phenotypically in the form of lower levels of log phase cell growth relative to WT cultures.
2.2.2 Cell Harvesting and Chromatophore Preparation. Cells were harvested by concentrating the culture to ~1-2L using a cell concentrator. Using a refrigerated centrifuge, the concentrated cell culture was centrifuged at 6,000 rpm for 15 - 20 min. Cells were stored at -80°C before the French press process. After thawing, several crystals of deoxyribonuclease and ribonuclease (Sigma), and 10 mM MgCl₂ were added to catalyze digestion of polynucleic acids released on cell disruption, so as to prevent their high viscosity from interfering with subsequent steps. The cells were kept on ice prior to disruption. Cells were disrupted by extrusion from a chilled French press cylinder at 850 psi (reading gauge at high position). The disrupted cells were chilled until the following procedures:

Unbroken cells were removed by centrifugation at 26,892 x g (12,000 rpm) for 20 min in a refrigerated centrifuge. The supernatant was decanted to new centrifuge tubes and centrifuged at 17,000 rpm for 40 min to spin down larger debris. Chromatophores in the supernatant were isolated by ultracentrifugation at 118,000 x g (40,000 rpm) for 90 min at 10°C. The pellet (chromatophores) was resuspended using a fine-hair brush in 50 mM MOPS pH 8.0 and 100 mM KCl buffer and diluted to ~twice the starting volume for washing. Washed chromatophores were again isolated by centrifugation at 118,000 x g (40,000 rpm) for 90 min at 10°C. Chromatophores were again resuspended in a minimum amount of 50 mM MOPS pH 8.0 with 10%-50% glycerol and 100 mM KCl buffer using a fine-hair brush. The chromatophore sample was stored at -80°C.

Purification of the intact $bc_1$ complex was conducted as in (44), with modifications. Isolated chromatophores were suspended in 50mM MOPS, 100mM NaCl, 1mM MgSO₄ pH 8.0 with 10% glycerol and 0.1% DDM (Buffer B), and gently passed through a chilled cell homogenizer. The concentration of heme $b$ was assayed at $\Delta A_{562-577}$ in the dithionite-reduced minus ferricyanide oxidized difference spectrum, and brought to a value of 1.2 through dilution with Buffer B.

For every 15ml of diluted chromatophore suspension, 10.5ml of Ni-NTA resin (~50% resin, ~50% buffer B) was added followed by 6ml of buffer B. Protease inhibitors PMSF (Phenylmethylsulfonyl fluoride) and E-ACA (E-Amino-n-Cuproic Acid) were used at 200µM and 100µM, respectively. In order to solubilize the $bc_1$ complex from the chromatophore membrane, the final solution was brought to 0.9% DM through addition from a 10% DM stock solution, and incubated with either gentle stirring or shaking for 45 minutes at 8°C. Incubated chromatophore/Ni-NTA slurry was loaded to a column and washed with buffer B containing 5mM histidine. The $bc_1$ complex was eluted with buffer B containing 200mM histidine.
Eluted bc$_1$ complex was dialyzed against buffer B to remove all L-His over 24 hours in two rounds of 50-100X dilution. Dialyzed bc$_1$ was concentrated to 10-30µM using an Amicon 50K centrifugal filter.

2.2.3 Preparation of cryobath mix-hold freeze-quenched EPR samples. Isolated bc$_1$ complex at 20 µM from wild type or E295W chromatophores was incubated with 200 µM oxidized equine cytochrome c, 20 µM ferricyanide, and 100 µM antimycin A (to inhibit electron transfer from heme b$_{H}$, and to prevent formation of SQ$_i$), with and without the Q$_o$-site inhibitor ascochlorin at 80 µM. Absorption spectra confirmed that the bc$_1$ complex and equine cytochrome c were oxidized prior to mixing (data not shown). To initiate the reaction, the bc$_1$ complex was mixed with 100 µM decyl-QH$_2$ in a 1:1 volume ratio, to final concentrations of 10 and 50 µM respectively. Both the bc$_1$ complex and decyl-QH$_2$ were prepared in a buffer of 50 mM MOPS at pH 8.5, 100 mM KCl, 20% glycerol, and 25 µg/ml 1-palmitoyl-2-oleoyl- glycerol-3-phosphocholine (POPC). Decyl-QH$_2$ was prepared from decyl-Q using borohydride reduction followed by cyclohexane extraction, and was diluted into degassed buffer immediately prior to use. Samples were mixed using a Biologic MPS-20 freeze-quench apparatus, housed in an anaerobic box equipped with gloves to allow manipulations prior to mixing. Before each experiment, the MPS-20 flow-lines were cleared with 250 µl of sample, which was removed by suction. All buffers and samples were degassed under argon for a minimum of 20 minutes before use in the anaerobic chamber. The reaction was started by mixing 250 µl of each solution, and quenched after 50 ms. The average freezing time of 35ms was determined from control studies if the kinetics of reaction of myoglobin with sodium azide (45). This, combined with mixing time and calculated time of flight, gave a total dead time of 50ms. Samples were injected directly into liquid pentane at −125° C, and then sealed and stored in liquid nitrogen. Following collection and freezing, the vials containing the frozen samples were thawed in a bath of liquid hexane brought to −60° C through immersion in liquid nitrogen in a secondary container. Frozen samples were packed into ~8 mm OD EPR tubes using stainless steel rods through a Teflon funnel immersed in hexane at −60° C. The sample volume in all cases allowed a filling of the EPR tube to a height > 2 cm, the maximum height of sample detection. Spin concentration of the SQ was determined using an internal standard (~2 µM Mn$^{2+}$ in final sample), to compensate for varia-
tions in packing density, and fluctuations in detector sensitivity over time, and then quantified by double-integration of the EPR spectrum and comparison against a ladder of external Mn$^{2+}$ standards. Spectra were measured at 1.0 mW power, 5.0 gauss modulation amplitude, 100 kHz modulation frequency, centered at 3320 gauss with a sweep of 160 gauss, effective amplifier gain of $10^4$, using microwave frequency ~9.30 GHz, with the value recorded for each measurement to allow calculation of $g$-values. Traces are the average of 75 scans of 25 s each.

2.3 Microfluidic Jet Development

In collaboration with Kenis Lab, we have developed a disposable jet-forming microfluidic chip for use as a rapid mixing device in mix/freeze-quench experiments. This chip is designed to eject microliter-scale volumes of solution at very high speeds to allow for off-chip analysis within micro-seconds of a reaction initiated in the mixing chamber of the chip. This device allows mixing and sample collection ~1000-fold faster using than current commercial apparatus. Previously published micron-jet forming devices have been fabricated from silicon or metal materials to meet high pressure requirements. These published designs are effective, but are expensive and require expertise to fabricate, making them inaccessible to many labs. Our design has a simple fabrication protocol with roughly a thousand-fold lower cost than published designs of similar performance, thus increasing the accessibility of this technique.

As stated above, most published designs either use silicon or metal fabrication strong enough to allow for very high chip pressure/solution speed, or an external pressurized air flow to initiate/maintain the jet. Silicon or metal fabrication is costly and requires expertise that is prohibitive to most labs, and an external pressurized air flow is not practical for freeze quenching applications. Therefore, we required a design that would flow the solution fast enough to initiate on-chip mixing and form a jet while keeping an internal pressure below 1MPa.

Several physical factors had to be balanced in order to achieve a stable jet at speeds needed for on-chip mixing with a PDMS-base chip. Even on a theoretical level, the physics of fluid dynamics in micron scale volumes at high speeds are not well characterized. Therefore, available fluid-dynamics equations could only be treated as guidelines on which we based our experiments to find the ideal jet-forming parameters. Most published microfluidics experiments with PDMS-glass or COC-PDMS-COC fabrication are conducted at low speeds where internal pressure is not
a large concern, and most known liquid jet-forming apparatuses are designed for millimeter scale or higher jet diameters where solution friction with the walls is a relatively small concern. We therefore had to experimentally optimize both internal pressure and solution friction to design a high-speed micron-scale jet within PDMS tolerance levels. The factors to be dealt with included (A) the construction materials, (B) the internal dimensions of the channels, (C) the outer dimensions of the exit nozzle, (D) the apparent outer dimensions of the exit nozzle, and (E) the flow speed of solution. All of these factors had to optimized in unison to allow us to form a stable micron-jet from inexpensive easy to fabricate materials. These parameters are generally discussed through use of the Darcy-Weisbach \((eq. \, 1)\), Reynold’s number \((eq. \, 2)\), and Weber number \((eq. \, 3)\) equations:

\[
\begin{align*}
\text{Eq. 1} \quad \Delta p &= f_D \cdot \frac{L}{D} \cdot \frac{\rho V^2}{2} \\
\text{Eq. 2} \quad R_e &= \frac{\rho V D_H}{\mu} \\
\text{Eq. 3} \quad W_{e_c} &= 4 \frac{B_0}{B_0} \left[ 1 + KB_{0}B_{0} - \left( (1 + KB_{0}B_{0})^2 - 1 \right)^{\frac{1}{2}} \right]^2
\end{align*}
\]

\(Eqs. \, 1 \, and \, 2\) allowed us to estimate the internal pressure within the chip, based on diameter \((D)\), length \((L)\), velocity \((V)\), solution density \((\rho)\) and dynamic viscosity \((\mu)\). The calculations were all treated as approximate since they were designed for pipes with a circular cross-section while our cross-section is rectangular (20 by 26 microns). In addition, the relatively poorly understood physics of turbulence/friction against the inner wall of a channel becomes very pronounced in a high speed microfluidic system where a significant percentage of the solution is interacting with the walls. We selected an upper limit of \(\Delta p\) as 1 megapascal, which is the pressure value at which tolerances have plateaued in published studies on PDMS microfluidic pressures.

The solution velocity must be kept as low as possible while allowing for jet formation and proper mixing of sample, since the pressure drop is proportional to solution velocity squared. We therefore used the mixing chamber design described in Li et al 2011(46), and demonstrated in Figure 2.4, where proper mixing with velocities as low as 8 \(\mu\)l/s are achieved.

We determined that internal pressure tolerance can be increased while simultaneously de-
creasing the Darcy-Weisbach friction factor by switching from COC-PDMS-COC to a PDMS-glass based fabrication. This single-layer design is stronger, and the glass has lower friction with the solution compared to the COC or PDMS. However, the PDMS-glass fabrication increases the chip outer diameter from ~220 microns to a centimeter. Application of eq. 3 would suggest that the increase of the Weber number (eq. 3) by increasing \( B_{o} \) would prevent jet formation due to beading on the nozzle. From these equations, with the glass-PDMS method using our calculated dimensions and a conventional nozzle, the flow speed would be fast enough for mixing, and fast enough to maintain a jet, but due to the high outer nozzle diameter, the flow speed would not be fast enough to initiate the formation of the jet. Notwithstanding this theoretical limit, we were able to initiate jet formation by using a wicking element at the exit nozzle. By continually wicking away exiting solution from the exit nozzle during jet initiation, the effective outer diameter of the nozzle is reduced to almost zero regardless of the actual outer diameter. This leads to jetting in the presence of ambient air-liquid interface with no pressure differential necessary. Once jetting is initiated, the wicking element can be removed without affecting the stability of jet formation.

Since the outer nozzle diameter was no longer a factor, we employed the PDMS-glass construction. It became necessary to align the PDMS to the edge of the glass to within ~10-20 microns. Manual alignment underneath a dissecting microscope proved to be significantly more effective than attempting a flush cut into the glass and PDMS surfaces.

Using this process, we are now able to generate a stable microfluidic jet of a mixed solution with ~1000 fold lower cost and greater ease of fabrication than currently published methods. This increases the accessibility of this technology in analytical devices.

We are aware of no prior publication of a wicking element for jet formation in the literature or in patents, nor of prior discussion of optimizing flow speed and channel dimensions against internal pressure in micron-scale jet formation. These processes together have allowed us to design the first ever PDMS-based jet-forming microfluidic chip that functions in the absence of an external pressure drop or air flow.

### 2.3.1 PDMS-Glass Microfluidic Chip Fabrication

RTV-PDMS was mixed thoroughly 1:10 with cross-linker and placed under vacuum until all visible air bubbles were removed. De-
gassed PDMS mixture was poured on top of silicon mold chips and incubated at 65°C for two hours. The PDMS containing the mixing device features was cut away from the mold. The exit channels were then cut to length ~20-50 μm. 100 μm-thick glass coverslip backings were thoroughly washed with alconox to remove any trace oil from the manufacturer.

SU-8-25 photoresist spin coating for 26μm depth channels:

<table>
<thead>
<tr>
<th>Step#</th>
<th>Ramp</th>
<th>speed</th>
<th>dwell</th>
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<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>500</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>2000</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>0</td>
<td>0</td>
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Silicon chips for the molds were spin-coated with SU-8-25 as described above. They were then placed on a 65°C hot plate for two minutes followed by a 95°C hot plate for 7 minutes, and allowed to briefly cool before UV exposure for 25s to establish the pattern of the mold. UV exposure was followed by a one-minute 65°C bake, and then a three minute 95°C bake. All non-polymerized photoresist was removed by a 5-minute incubation in a PGMEA bath, followed by a PGMEA rinse, an isopropanol rinse, and drying by a nitrogen gas jet. Prepared molds were then silanized by placing them under vacuum in the presence of a silane reservoir for four hours.

2.3.2 Ultra-Fast Freeze Quenching Copper Wheel Apparatus. Design of the freeze-quenching copper wheel apparatus was based on that discussed in Zhu et al. (3) and Li et al. (46), with modifications. Oxygen-free copper wheels were mounted in an oxygen-free copper housing to prevent disproportional changes in dimensions from thermal contraction. Wheels were initially spaced to be touching on-edge, and then sanded down by ~100-200 μm. As an improvement on the published design, a 1:1 gearing was incorporated on axis on both wheels to ensure synchronized counter-rotation. This overcame the unreliability of sample friction alone to drive the second wheel. The copper housing was set in a Teflon base, which allowed it to be partially immersed in the N₂(l), contained in Dewar flask. The wheels were spun at ~300rpm through a DeWalt 24V hammerdrill motor and gearbox.

The wheels were buffed and polished before each experiment to remove trace buildup of oxi-
dized copper. In addition to keeping the wheels partially submerged in N\textsubscript{2}(l) during sample collection, the entire housing with the Teflon base was removed and fully submerged in N\textsubscript{2}(l) for several minutes in between each sample.

As discussed in Manzerova et al. (45), there have been no reported instances in the literature of an oxygen-free copper-based freeze quencher leading to trace copper signal within EPR sample. The only reported instance of contamination did not specify that oxygen free copper was used in the wheels. Any trace copper metal fragments from the wheels would be EPR silent since metallic copper is magnetically transparent (45).

2.3.3 Freeze Quenching Calibration. The standard myoglobin sodium azide reaction was used to determine freezing time of our ultra-fast microfluidic freeze quench mixer as in Appleyard et al. 1994 (45). The myoglobin (250µM), and sodium azide (25mM) were mixed in a 1:1 ratio in the microfluidic chamber, and the reaction was monitored through the disappearing EPR signal of oxidized myoglobin. Plotting the ln of intensity (I) versus time of flight allows us to extrapolate backwards to the time at ln(I) =0, when the reaction has just begun (Fig 2.1). This time at ln(I) =0 represents the dead time; the sum of the time to exit the chip, and the time of freezing, with the remainder of the time being controlled time of flight. Points were taken at 1.0, 1.7, and 10.0 ms time of flight with fully oxidized myoglobin signal determined through myoglobin solution without azide. The broad trough centered at g=6 observed in Cherepanov et al. (37) independent of azide binding under similar conditions, was used as an internal standard. Our calculated freezing time is smaller than the standard deviation of our calibration, which results in a theoretical calculated time of freezing of less than zero. (See Fig.2.1)

In all kinetic studies reported here, we have assumed a freezing time of zero, and total dead time of 30 µs, which is the time to exit the chip after mixing is initiated. The 50µs calculated dead time of the similar microfluidic freeze quenching device of Lin et al. is in close agreement with this.

Due to variation in the angle of exit of the jet, positioning of the microfluidic chip was not accurate enough to define the time of flight within an error of ±50µs. However, for the purposes of experiments with multiple time-points over several milliseconds, this inaccuracy does not invalidate conclusions based on observed trends or parameters.
2.4 Ultrafast Microfluidic Freeze Quenching

Our assays used a protocol similar to that of Zhu et al (3), but exploring a wider range of initial conditions. Unless otherwise indicated, all assays were performed in the presence of antimycin, and explored the kinetics of SQ\textsubscript{o}, identified by sensitivity to Q\textsubscript{o}-site inhibitor ascochlorin.

The SQ\textsubscript{o} species was assayed through X-band CW EPR spectroscopy of the paramagnetic state observed in the g=2.00 region. Samples were frozen at different times after mixing of either bc\textsubscript{1} complex either partially or fully reduced in the presence of excess OH-decylUQH\textsubscript{2}, with excess equine ferricyt c as oxidant, or by mixing the bc\textsubscript{1} complex, fully oxidized by excess equine ferricyt c, with excess OH-decylUQH\textsubscript{2}. Microfluidic ultra-fast freeze quenching was used to acquire samples at time points ranging from 166\textmu s to 4.0ms.

Different initial redox states were chosen so as to vary the number of oxidizing equivalents available in the antimycin-inhibited low-potential chain, as indicated by the redox state of the b-hemes. States studied were: (i) with the chain fully oxidized, \textit{b}\textsubscript{H}(ox)\textit{b}\textsubscript{L}(ox); (ii) with the chain half reduced, \textit{b}\textsubscript{H}(red)\textit{b}\textsubscript{L}(ox); and (iii) with the chain fully reduced, \textit{b}\textsubscript{H}(red)\textit{b}\textsubscript{L}(red), with 2, 1 or 0 equivalents available as acceptor for the electron from SQ\textsubscript{o}, respectively, in (i), (ii) or (iii). Kinetics under all three conditions were studied using native bc\textsubscript{1} complex in WT alone. Kinetics were also studied in the fully oxidized and \textit{b}\textsubscript{H} reduced complex using bc\textsubscript{1} complex isolated from strain E295Q.

With the initially oxidized (state \textit{b}\textsubscript{H}(ox)\textit{b}\textsubscript{L}(ox)), bc\textsubscript{1} complex at 6.2 \textmu M heme \textit{c}\textsubscript{1} was pre-incubated with 800 \textmu M equine ferricyt c acceptor in the presence of antimycin A under anaerobic conditions, and held in one syringe. The other syringe contained 65\textmu M of OH-decylUQH\textsubscript{2}, generated by pre-incubation of OH-decylUQ with 0.5 mM NADH in the presence of 70 nM NDH-2 to catalyze the NADH-UQ reductase reaction. The NADH mix was initially placed in the bulb of a modified Thunberg cuvette, and added to the NDH-2/OH-decylUQ quinone mix in the main compartment after two hours under argon to ensure anaerobiosis. After mixing of the NADH and quinone pool, they were allowed to react for another half hour to come to fully reduce the quinone pool, before transfer to the syringe.

With the initially half-reduced state (\textit{b}\textsubscript{H}(red)\textit{b}\textsubscript{L}(ox)), bc\textsubscript{1} complex in the presence of antimycin A, the 65\textmu M quinone pool and NDH-2 was flushed with argon for 2 hours before mixing with 0.5 mM NADH held in the bulb of the anaerobic Thunberg cuvette, and allowed to incubate
for 30 minutes. The redox state of the b-hemes was monitored spectrophotometrically; somewhat remarkably, heme $b_H$ became reduced in parallel with reduction of the Q-pool, but heme $b_L$ remained oxidized, despite the strongly reducing conditions provided by the excess NADH ($E_h < -350$ mV). In a separate anaerobic chamber, 800µM equine ferricyt c was flushed with argon to maintain anaerobiosis. The reaction progress was assayed my rapid-mix, freeze-quench after transfer of aliquots to the reaction syringes, with the redox state of the reactants monitored through optical spectra collected between each sample. Because the failure rate of the rapid mix chips was high, sample collection for a complete set of kinetic points sometimes had to involve different batches, in which the initial $bc_1$ concentration varied. The $bc_1$ complex concentrations in the premixed syringe were as follows: 7.33µM for data collected at 2ms and 4ms, and 11µM for data collected at 0.25ms and 0.5ms, and for the 0 time point sample.

For the fully reduced state ($b_H$(red)$b_L$(red)), sample preparation was essentially the same as for the $b_H$(red)$b_L$(ox) state, except that 30µl of freshly prepared 1mg/ml dithionite was added to the 3ml sample to fully reduce the $bc_1$ complex, and antimycin A was absent.

The studies using $bc_1$ complex from strain E295Q were prepared as for the native complex. For $b_H$ reduced only, 6.2µM $bc_1$ complex was used. For the fully oxidized complex, 6.2µM was used for data collected at 0.38 and 0.78ms, and 3.6µM for data collected at 0.16 and 1.53ms.

The concentration of $bc_1$ complex was assayed spectrophotometrically from the difference at 551-542 nm for [cyt c1], and was between 3.6µM and 11µM, NDH-2 was at 70nM, with 65µM OH-decylUQ, 500µM NADH, and 800µM equine cyt c. Concentrations of semiquinone were assumed to change in proportion to the amplitude of the EPR spectrum.

**Occupancy of SQ$_0$ state**

The intensities of the SQ$_0$ state shown on the Figures were taken from the amplitudes of the CW spectra after normalization to take account of the $bc_1$ complex concentration after mixing, and should be taken as indicating relative rather than absolute values. The signal amplitudes are considerably higher than expected from a spin $\frac{1}{2}$ paramagnetic free-radical state, likely because of spin sharing with a neighboring center. Unfortunately, without knowledge of the spin quantum number of that state, quantification of the spin amplitude is not possible. Power, gain, sample concentration, sample height, and temperature were all factored in comparing signal intensities.
Unless otherwise noted, spectra were collected at 77K. Due to poor signal to noise, the time course in some samples taken using \(bc_1\) complex from the E295Q strain in the \(bH(\text{red})bL(\text{ox})\) state had to be taken from spectra measured at 15K. The signal intensity of these data was re-scaled to allow comparison with data measured at 77K by using temperature dependence monitored in the E295Q 250\(\mu\)s sample as a calibration curve. Daily intensity fluctuation in our CW EPR instrumentation was controlled for by measuring the same sample on multiple days.

For measurement in all kinetic experiments, power was kept at 0.2mW to prevent signal saturation unless otherwise indicated (for example in power saturation assays). Modulations of 2.0 and 4.0 were used, field center was kept at 3320 gauss with a sweep of 400 gauss, at microwave frequency ~9.25 G Hz, with exact values determined for each run.

### 2.4.1 Pulsed EPR

EPR measurements were carried out using a Varian E-line 12" Century Series for X-band CW EPR, and a Bruker Elexsys E580 spectrometer for X-band ESEEM, both equipped with Oxford CF 935 cryostats. Liquid helium was used at variable flow rates to adjust the temperature from 5K to 70K. Pulsed EPR data collection and analysis was performed in close collaboration with Dr. Sergei Dikanov, and CW X-band data in collaboration with Dr. Mark Nilges.

In the two-pulse experiment (\(\pi/2-\tau-\pi-\tau-\text{echo}\)), the intensity of the echo signal is measured as a function of the time interval \(\tau\) between two microwave pulses with turning angles \(\pi/2\) and \(\pi\) to generate an echo envelope that maps the time course of relaxation of the spin system (in ESEEM) or as a function of magnetic field at fixed \(\tau\) (in field-sweep ESE).

For single-\(\tau\) ESEEM experiments, values of \(\tau\) were scanned iteratively to determine \(\tau\) values with optimal signal:noise before data collection over a larger average. ESEEM spectra from experiments using WT \(bc_1\) complex were measured at 120ns, 15K. 120ns decayed sample was measured at 35K. For experiments with E295Q complex, the ESEEM plot was measured at a \(\tau\) value of 136ns at 29K.

### 2.4.2 NDH-2

For the ultra-fast freeze quenching assays, NADH-quinone oxidoreductase (NDH-2) of \(E.\ coli\) was used at 70nM with 500\(\mu\)M NADH instead of the direct addition of chemically reduced QH\(_2\) as used in Cape et al. (4), Zhu et al. (3), and Victoria et al. (2). Enzy-
matic quinone reduction ensured a stable fully reduced quinol pool even in the event of trace oxygen contamination due to the ability of the NDH-2 to re-reduce any quinol that reacts with trace amounts of oxygen. At 70 nM, any radical signal from the NDH-2 is below the detectable signal to noise of our instrumentation. In addition, the higher aqueous solubility of idebenone (OH-decylUQH$_2$) gave it very poor yields for chemical reduction followed by organic phase extraction; making enzymatic reduction a necessity.

2.4.3 OH-decylUQH$_2$ Substrate. The choice of substrate quinol for $bc_1$ complex kinetic studies has been important in attempting to reproduce in vitro the kinetics observed in situ in chromatophores. In the chromatophore, UQ-10 partitions entirely to the membrane, with the isoprenyl$_{10}$ tail in the lipid phase, and is in >30-fold excess over the complex, which has a membrane concentration ~0.5 mM. Experiments in vitro involve the isolated complex in micellar dispersion solubilized by detergent in a predominantly aqueous medium. The primary issue with the commonly used decylubiquinol substrate has been its very low critical micellar concentration, below ~30 µM, which limits the available concentration even when excess is added. This strongly constrains what concentration of the $bc_1$ complex can be used, because the quinol substrate has to be added in excess to allow for multiple turnovers. An alternative has been to synthesize substituted decylUQ variants, for example with a halogen at the free end of the decyl chain.

During the last few years, a more convenient substrate, idebenone, OH-decylUQ, with the substituent at the free end of the decyl sidechain, has become available as a low-cost commercially available alternative. Idebenone is identical to decylubiquinone except for a single –OH group on the final carbon of its 10-carbon tail (see Fig 2.6). This has the advantage of increasing solubility so that higher available concentrations can be achieved, and therefore high [$bc_1$ complex] used. We have therefore used OH-decylUQ in place of decylUQ in our assays.

2.5 Kinetic model

The kinetic model is described in detail by Victoria et al. (2), who also provided full scripts for running simulations under the Dynafit program (66) for a model with $b_H$ and $b_L$ initially oxidized. The full scripts for models with (i) $b_H$ reduced and $b_L$ oxidized, and (ii) both hemes reduced at time = 0 are provided below.
We note here that the kinetic model was based on parameters determined for the kinetic and thermodynamic behavior in situ in chromatophore membrane-bound system, with kinetics initiated by flash-activation of the reaction center to provide the substrates for the bc\textsubscript{1} complex. In the model, occupancy of intermediate states is constrained by well-characterized thermodynamic parameters that likely pertain to the in vitro system studied here. However, kinetic parameters involving external substrates, might be expected to need modification. As will be apparent from the following chapters, the model will also need to be refined to take account of kinetic and thermodynamic parameters associated with possible formation of a complex between SQ\textsubscript{o} and ISPH revealed in this work. Because thermodynamic parameters constrain the occupancy of total SQ\textsubscript{o} species, we believe that the current model should provide useful values through which discussion can be framed. Since the kinetic parameters govern rates of transition between states, but are constrained by observed kinetics, it is likely that the model also provides a useful guide with respect to such features. However, we have made no attempt to use the Dynafit program to model the kinetics measured, since such an exercise would be futile until the model itself is revised. A further important constraint comes from the lack of parameters through which absolute occupancies can be determined from the data available. In using the model to predict outcomes with which to compare our experimental data, we have kept all parameters the same as used in the published account. However, we have taken account of different ratios between substrate and complex in adjusting to an in vitro soluble system with idebenone and equine cytochrome c as external substrates.
Fig. 2.1 Ultra-fast freeze quenching calibration; MbOH reaction with sodium azide plotted as the log of the percent intensity over time in ms. 387\(\mu\)M Myoglobin reacted 1:1 with 100mM azide.
Fig. 2.2 Sample spectrum of anaerobic $bc_1$ complex preincubated with $\sim 70\text{nM}$ NDH-2 and $65\mu\text{M}$ OH-decylQH$_2$. NADH solution was added following deoxygenation to prevent unnecessary NADH consumption from OH-decylQH$_2$ reacting with oxygen. The above spectra was taken 30 minutes after addition of NADH, and demonstrates $bc_1$ complex held stably in the $b_1(\text{ox})b_1(\text{red})$ redox state until initiation of the reaction by mixing with an anaerobic solution of excess equine cyt $c$ acceptor ($400\mu\text{M}$).

![Diagram](image)

Fig. 2.3 A microfluidic jetting device mixes reactants within microseconds, and ejects a 25 micron jet moving $15\text{ m/s}$ onto spinning copper wheels immersed in liquid nitrogen for a total minimum dead time in the dozens of microseconds.
Fig 2.4 High contrast image acquired through a dissection microscope of a microfluidic chip flowing solutions of water premixed with either red or green dye. The solution is completely mixed with ~30µs needed to cross the entire length of the mixing channel and exit the device.
Fig. 2.5 Microfluidic freeze quench apparatus. (A), copper wheel freeze quenching device, (B), microfluidic jet under magnification, (C) jetting microfluidic mixer chip under magnification.
Dynafit Script for Kinetics of the \( bc_1 \) complex; \( b_H \) reduced at time = 0

(task)

data = progress

[mechanism]

;\( E \) is enzyme, \( I \) is \([\text{FeS}_2] \), \( c \) is heme \( c_1 \), \( L \) is heme \( b_L \), \( H \) is heme \( b_H \);
;Q, QH, and QH2 are quinone and quinol forms, \( Q_d \) and \( Q_p \) in distal and proximal domains,
;\( SQ_d \) and \( SQ_p \) are SQ intermediates in distal and proximal domains,
;redox metal centers are indicated by a minus sign (-).
;The simulation is inhibited (implicitly) by antimycin.

;By default, the simulation allows 1 turnover of the Qo-site, and the progress is displayed
;through reduction of heme \( b_H \) (see first line in [response] section).

;First turnover

\[ E-c-L-H- + Aox \iff E-c-L-H- + Ared : k_3 \iff c_1 -e-> Aox \]
\[ E-c-L-H- + QH_2 \iff E-c-L-H-.QH_2 : konQH koff \iff \text{formation of ES-complex (assumes I is oxidized)} \]
\[ E-c-L-H-.QH-H \iff E-c-L-H-.QH-H : kproton k-proton \iff \text{proton distribution in } H-bond \]
\[ E-c-L-H-.SQ_d \iff E-c-L-H-.SQ_d : k_1 k-1 \iff QH_2 ox. - electron transfer reaction \]
\[ E-c-L-H-.SQ_d + Aox \iff E-c-L-H-.SQ_d + Ared : k_3 k-3 \iff c_1 -e-> Aox \]
\[ E-c-L-H-.SQ_d \iff E-c-L-H-.SQ_d : k_2d k-2d \iff SQ -e-> bl from distal pocket \]
\[ E-c-L-H-.SQ_d \iff E-c-L-H-.SQ_d : kdiff kdiff \iff \text{diffusion of SQ in pocket} \]
\[ E-c-L-H-.SQ_d \iff E-c-L-H-.SQ_d : k_{2p} k_{2p} \iff SQ -e-> bl from proximal pocket \]

;we assume Qi-site Locked so bH remains reduced
\[ E-c-L-.H-.Qp \iff E-c-L-.H-.Qd : kdiff kdiff \iff \text{diffusion of Q to distal domain} \]
\[ E-c-L-.H-.Qp \iff E-c-L-.H-.Qd : k_{2p} k_{2p} \iff \text{equilibration of Q QH2} \]

;Now we have to deal with alternative hipot chain states
\[ E-c-L-.H-.SQ_d \iff E-c-L-.H-.Qd : k_{2d} k_{2d} \iff SQ -e-> bl from distal pocket \]
\[ E-c-L-.H-.SQ_d \iff E-c-L-.H-.SQp : kdiff kdiff \iff \text{diffusion of SQ in pocket} \]
\[ E-c-L-.H-.SQ_d \iff E-c-L-.H-.SQp : k_{2p} k_{2p} \iff SQ -e-> bl from proximal pocket \]

;we assume Qi-site blocked so bH remains reduced
\[ E-c-L-.H-.Qp \iff E-c-L-.H-.Qd : kdiff kdiff \iff \text{diffusion of Q to distal domain} \]
\[ E-c-L-.H-.Qp \iff E-c-L-.H-.Qd : k_{2p} k_{2p} \iff \text{equilibration of Q QH2} \]

;Second turnover

\[ E-c-L-.H- + QH_2 \iff E-c-L-.H-.QH_2 : konQH koff \iff \text{redox state of bH, bL does not affect dissociation of QH2} \]
\[ E-c-L-.H-.QH_2 \iff E-c-L-.H-.QH-H : kproton k-proton \iff \text{proton distribution in } H-bond \]
\[ E-c-L-.H-.QH-H \iff E-c-L-.H-.SQ_d : k_1 k-1 \iff QH_2 ox. - electron transfer reaction \]
\[ E-c-L-.H-.SQ_d + Aox \iff E-c-L-.H-.SQ_d + Ared : k_3 k-3 \iff c_1 -e-> Aox \]

45
EI.c.L-.H-.SQd <=> EI.c.L-.H-.SQp ; kdiff3 k-diff3 ;If bL is charged coulombic repulsion will change K

[constants]

konQH = 500 ;K(bind) is given by kon[QH2]/koff, K= 10 at QH2=100
koff = 500
konQ = 50 ;K=1 at Q=100; i.e. konQH = 10.konQ when pool half-reduced
diff = 10000000 ;proton difference of 5 in ES (pKESF=6.5;pWHQ2=11.5); K = 10^-5
dataQH2 = 221000000 ;proton transfer down H-bond of 10^-12/s
k1 = 430000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k2 = 430000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff = 10000000 ;default is 10^7 /s; plausible values are in the range < 10^9 /s
kproton = 10000000 ;pK difference of 5 in ES (pKISP=6.5;pKQH2=11.5); K = 10^-5
k-QH = 50 ;K=1 at Q=100; i.e. konQH = 10.konQ when pool half-reduced
koff = 5000000 ;proton difference of 5 in ES (pKESF=6.5;pWHQ2=11.5); K = 10^-5
k-Q = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-QH2 = 430000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k-diff3 = 1000000000000 ;Marcus rate for R = 7A, lambda 0.8V, deltaG -0.1V
k-diff3 = 1000000000000 ;Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V

[rules]

EI.c.L-.H-.SQd <=> EI.c.L-.H-.SQp ; kdiff3 k-diff3 ;If bL is charged coulombic repulsion will change K

[responses]

;the first line allows progress to be followed through reduction of the high potential acceptor
Ared = 1 ;reduced hipotential acceptor

;uncomment these lines for bh
;EI.c.L-.H-.Qp = 1
;EI.c.L-.H-.Qd = 1
;EI.c.L-.Q = 1
;EI.c.L-.H-.QH2 = 1
;EI-.c.L-.H-.Qp = 1
;EI-.c.L-.H-.Qd = 1
;EI-.c.L-.H-.Q = 1
;EI-.c.L-.H-.QH2 = 1

;uncomment these lines for fully reduced complex
;EI.c.L-.H-.Qp = 1 ;fully reduced complex as product measured.
;EI.c.L-.H-.Qd = 1 ;Setting response for all species at 1 means
;EI.c.L-.H-.Q = 1 ;we get the sum of these as response curve.
;EI.c.L-.H-.QH2 = 1 ;First 2 are transients, and last shows lag

[concentrations]

EI.c.L-.H- = 1
QH2 = 200
Aox = 6

[progress]

mesh linear from 0 to 0.01 step 0.00001
monitor EI.c.L-.H-.SQd, EI.c.L-.H-.SQp, EI-.c.L-.H-.SQd, EI-.c.L-.H-.SQp, EI-.c.L-.H-.SQd, EI-.c.L-.H-.SQp ;early SQ intermediates
monitor EI.c.L-.H-.SQd, EI.c.L-.H-.SQp ;SQ intermediate in distal domain, SQ intermediate in proximal domain in TO3
directory ./data/simulate/11-10-11 ;the ./ means file directory containing Dynafit.exe
time kdiff7-A2-c.txt

[output]

directory ./data/output/simulate/progress

Dynafit Script for Kinetics of the bc1 complex; fully reduced at time = 0

[task]
task = simulate
data = progress

[mechanism]

:E is enzyme, I is [2Fe2S], c is heme c1, L is heme bL, H is heme bH,

;Q,QH2 are quinone and quinol forms, Qd and Qp in distal and proximal domains,
;SQd and SQp are SQ intermediates in distal and proximal domains, 
; reduced metal centers are indicated by a minus sign (-).
; The complex is inhibited (implicitly) by antimycin.
; By default, the simulation allows 2 turnovers of the Qo-site, and the progress is displayed
; through reduction of the high potential acceptor (see first line in [response] section).
; To simulate the first turnover only,
; comment out the set of states after comment line Second turnover below
; and select display of heme bH from the [response] section

; Partial turnover to generate SQ when low potential chain acceptors are pre-reduced.
EI.c.L-H + QH2 <=> EI.c.L-H.QH2 : konQH koff ; reduced state of bH, bL does not affect dissoc. of QH2
EI.c.L-H.QH-H <-> EI.c.L-H-Qd : k1 k-1 ; QH2 ox. - electron transfer reaction
EI.c.L-H-Qd + Aox <-> EI.c.L-H-Qd + Ared : k3 k-3 ; c1 e- > Aox
EI.c.L-H-Qd <-> EI.c.L-H-Qd : kdiff3 k-diff3 ; If bL is charged coulombic repulsion will change K

[constants]
konQH = 500 ; K(bind) is given by kon[QH2]/koff, K~ 10 at QH2=100
koff = 5000
konQ = 50 ; K=1 at Q=100; i.e. konQH = 10.konQ when pool half-reduced
kdiff = 100000000 ; default is 10^7 /s; plausible values are in the range < 10^9 /s
kproton = 100000000 ; pK difference of 5 in ES (pKISP~6.5; pKQH2=11.5); K~ 10^-5
kproton = 100000000000 ; proton transfer down H-bond of 10^-12/s
k1 = 221000000 ; Marcus rate for R = 7A, lambda 0.8V, deltaG ~ 0.1V
k2 = 43000000 ; K for this step of 5.1. K(1st electron) is 5.1 x 10^-5
k2p = 4300000000 ; Marcus rate for R = 6.9A, lambda 0.7V, deltaG=0.225V
k2d = 6880 ; to give K(2nd electron) = 6250.
kd = 1200 ; from occupancy .05, rate = 60/s; ratio gives same K(2nd electron)
kd = 0.192 ; K(overall) is then ~3.18, the same as from Em values
k = K(1st1), K(1st2)=10 x 5.1.10^-5 x 6250 = 3.18
kd = 100000000 ; k 10^7 /s
kd = 100000000 ; K=10
kd = 100000000 ; k 10^3 /s
kd = 6250 ; to give K=160, appropriate to 130 mV difference in Em
kd = 100000000 ; ISP e- -> c (includes dissoc from b-pos to c-pos)
k = 300000000 ; K=0.033
kdiff3 = 100000000 ; to give K= 0.01
k-diff3 = 100000000 ;

[responses]
the first line allows progress to be followed through reduction of the high potential acceptor
Ared = 1 ; reduced hipotential acceptor
the next 4 lines allow display of heme bH reduction during first turnover of the Qo-site. These are consumed on the second turnover
EI.c.L-H-Qp = 1 ; reduced heme H as product measured.
EI.c.L-H-Qd = 1 ; Setting response for all species with H at 1 means
EI.c.L-H- = 1 ; we get the sum of these as response curve.
EI.c.L-H-QH2 = 1 ; First 2 are transients, and last shows lag
the next 4 lines allow display of heme bL reduction when 2 turnovers are implemented
EI.c.L-H-Qp = 1 ; reduced heme H as product measured.
EI.c.L-H-Qd = 1 ; Setting response for all species with H at 1 means
EI.c.L-H- = 1 ; we get the sum of these as response curve.
EI.c.L-H-QH2 = 1 ; First 2 are transients, and last shows lag
By commenting out all but one set, you can see each individually
; because undefined responses are given coefficient 0 in assessing progress

[concentrations]
EI.c.L.-H = 1
QH2 = 200
Aox ~2 ; assume 2 equiv. of external acceptor with K=100 (at Em ~400 mV), to simulate high potential chain beyond I

; Change the stoichiometry to observe the increased SQ occupancy following a partial third turnover

[progress]
mesh linear from 0 to 0.01 step 0.00001
directory ./data/simulate/11-10-11 ; the ./ means file directory containing Dynafit.exe
file kdiff7-A2-c.txt
[output]
directory ./data/output/simulate-progress
[end]
Chapter III: RESULTS, Freeze Quenching Kinetics

3.1 Mix-hold Freeze Quenched EPR

Abstract

As demonstrated by Cape et al.(4) and in our own work (Victoria et al. (2)), the Q_o site SQ can be trapped at ~5-10% occupancy per monomer on initiating turnover by mixing the oxidized complex with quinol substrate when oxidation of heme b_H is prevented by antimycin A inhibition of the Q_i site. Because the high potential chain (heme c_1 and ISP) can be kept oxidized by excess cyt c acceptor, the b_L and b_H hemes of the low potential chain become fully reduced after two turnovers of the Q_o-site. Therefore, at the third turnover, the incoming quinol undergoes its first proton/electron transfer with the ISC, but then has nowhere to deposit its second electron, and the SQ radical intermediate builds up to the point of being visible to EPR. Classical freeze quenching methods for EPR sample preparation only allow for time points to be collected at 10ms and above. For wild type bc_1 complex, this is ten times longer than needed for one turnover. However, for mutant strain E295W, Victoria et al. (2) showed that the Q_i-site inhibited enzyme still has a mostly oxidized low potential chain for over 50 milliseconds because the mutation blocked reduction of heme b_L by SQ_o. This behavior in E295W was exploited to quantify both reaction partners, [SQ] and [ferriheme b_L], under conditions in which inhibited forward flux was occurring, and from this to determine an empirical rate constant for this reaction. The large bulky W side chain would be expected to confine the SQ to the distal region of the Q_o site, allowing us to directly calculate the rate constant from the distal region of the Q_o site. The rate constant, \( k \), is given by \( k = \frac{v}{([SQ].[ferriheme b_L])} \), with terms on the right provide the rate, \( v \), observed \textit{in situ}, and by the measured occupancies. At the observed SQ occupancy of ~0.06 per monomer, we calculate a rate constant three orders of magnitude smaller than what is required for a concerted model for the Q_o site mechanism; strongly supporting a diffusional model of electron transfer.
**Experimental**

Methods for the mix-hold cryobath-based freeze quenching are as published in (2), and are as described above. Briefly, Isolated $bc_1$ complex at 20 µM from wild type or E295W chromatophores was incubated with 200 µM oxidized equine cytochrome c, 20 µM ferricyanide, and 100 µM antimycin A (to inhibit electron transfer from heme $b_H$, and to prevent formation of SQ at the $Q_1$-site), with and without the $Q_O$-site inhibitor ascochlorin at 80 µM. To initiate the reaction, the $bc_1$ complex was mixed with 100 µM decyl-$QH_2$ in a 1:1 volume ratio, to final concentrations of 10 and 50 µM respectively. Both the $bc_1$ complex and decyl-$QH_2$ were prepared in a buffer of 50 mM MOPS at pH 8.5, 100 mM KCl, 20% glycerol, and 25 µg/ml 1-palmitoyl-2-oleoyl- glycero-3-phosphocholine (POPC).

All reactions were performed under anaerobic conditions in a glove box with a Biologic MPS-20 freeze-quench apparatus with -120°C liquid pentane. The apparatus was calibrated to a freeze-quench dead time of 35ms through a myoglobin-azide control reaction, for a total 50ms dead time including time of flight. Spin counting was performed with the kind assistance of Dr. Rimma Samoilova and was performed with a Mn$^{2+}$ standard ladder and a trace internal Mn$^{2+}$ concentration of ~2µM.

**Results:**

The $Q_0$ site SQ was first clearly observed by Cape et al. (4), since confirmed by Zhang (41), Victoria et al. (2) and Sarewicz et al. (26). Observed values for SQ occupancy of the $Q_0$-site vary widely, likely reflecting varying freeze-quenching methodologies on the millisecond to second timescales. No data for E295 mutants had been reported until our published work (2) where we used cryobath freeze quenching protocols similar to those reported in (4, 26). Here we describe the published (2) results of freeze-quenched WT and E295W $bc_1$ complex with trapped $Q_0$-site SQ intermediate, and discuss their significance in supporting the $Q_0$ site diffusional kinetic model.

Our WT results are consistent with those of Cape et al. (4); Fig. 3.1 shows the SQ signals after correction for the ascochlorin insensitive component (ascochlorin occupies the $Q_o$-site (42),
as well as inhibiting the $Q_i$ site, and is expected to displace all other occupants). The yields of SQ in our study, ~3% for WT and ~6% for E295W, were similar in both strains (though higher in the E295W), and at the high end of the range previously reported by Cape et al. (4). Although the subtracted spectra are somewhat noisy given the low signal to noise of a single $S=1/2$ system, the $g$-values (2.0061 in WT and 2.0058 in E295W) were, within the accuracy of the noise, similar to those determined in Cape et al (4), where chemical reduction of decylubiquinone generated $Q^-$, which had $g=2.0056$. Our line-widths, however, were both wider (24 in WT and 18 in E295W), perhaps because of some contaminating background signal that was not subtracted.

Our attempts to measure SQ generated through illumination of chromatophores in a manner analogous to Zhang et al. (28) but in a time-resolved freeze-quenching context were inconclusive. When the delay before measurement was short enough to leave a substantial driving force in the high-potential chain, the EPR signal due to residual $P^+$ swamped any potential SQ signal, as might be expected from the kinetics of decay of the optical signal for $P^+$ (28). The $P^+$ and SQ signals proved to be unresolvable through differences in power saturation, making clear observation of SQ signal impracticable.

While our calculated E295W occupancy was at the higher end of the range reported in (4), the occupancy value expected during the normal uninhibited wild type reaction (with acceptors available in both chains) would, of course, be much lower than under these forcing conditions and likely too low for direct measurement. Even under our higher SQ occupancies with E295W, at the concentration of $bc_1$ complex used, the uncoupled $S=1/2$ spin state [SQ] is at the low-end of signal detectable by our EPR apparatus (2).

**Discussion:**

In considering the concerted versus diffusional models of electron transfer we will assume initially a WT $bc_1$ occupancy of 0.01 SQ/Q$_O$-site, which is the smallest published [SQ] observed experimentally and is consistent with the limits from kinetic data. We will then use the SQ occupancy we measured to estimate an empirical value for the rate constant for oxidation of SQ in the distal domain by heme $b_1$, and from this derive other thermodynamic and kinetic parameters.

For calculation of electron transfer rates using the Marcus-Brønsted equation, with parameters: $\lambda = 0.7$ V, and $\Delta G^0' = -0.1$ V, assuming a classical Marcus treatment of the Arrhenius term
with $\gamma = 4.23$ (23), and assuming homogeneity of dependence of intrinsic rate constant on distance as discussed in [Moser, Dutton, 1995], the 11.5 Å for an edge-to-edge distance from an occupant of the distal domain to heme $b_L$ gives a theoretical value of the second electron transfer of $\sim 10^6 \text{s}^{-1}$. Meanwhile, the diffusional model provides a 6.5 Å distance from the proximal SQ to the $b_L$, which gives $\sim 10^9 \text{s}^{-1}$. These values are similar to those used in previous studies and discussions (31, 32, 33, 34). Are these theoretical values from these two models both consistent with the data?

The maximal rate measured in uninhibited chromatophores ($\sim 10^3 \text{e}^- / bc_1 / \text{s}^{-1}$ at pH 8), is determined by the rate constant for the first electron transfer with a saturated $ES$-complex (occupancy $\sim 1$). To at least match this rate for the second electron transfer using occupancy of 0.01, a minimal value for the rate constant would have to be in the range $10^5 \text{s}^{-1}$, which is compatible with the value derived from an 11.5 Å distance. Therefore, given the uncertainty of occupancy values from earlier experiments, a simple mechanism in which the SQ could donate electrons to heme $b_L$ from the distal domain could not be excluded based on the $k_{cat}$ at pH 8 alone.

Three other factors make this naïve scenario questionable: (i) the reaction is certainly not just a simple electron transfer, but would also involve transfer of the proton towards the water chain (32) (ii) the actual SQ occupancy under uninhibited flux in the WT complex must be much lower than the value of 0.01 observed under inhibited or mutant conditions, so likely the value used in the example above is a significant overestimate, artificially inflating the overall rate to favor the concerted model, and (iii) the intrinsic rate of removal of SQ would have to not only match, but substantially exceed the rate of generation in order to account for the dependence of observed rate on pH and driving force in mutants. The full effect from dramatic changes in $E_m$ for ISP (31, 35) is as expected from Marcus theory if the first electron transfer is consistently limiting. The lack of any consistent effect on the overall rate from changes in the $E_m$ for heme $b_L$ (for example, no enhanced rate when $E_m$ of heme $b_L$ is raised by $\sim 80 \text{mV}$) is consistent with a much faster intrinsic rate for the second electron transfer (41) compared to the first electron transfer.

Assuming a factor of ten for each of these last two points, we would need a value $> 10^7 \text{s}^{-1}$, raising the question of consistency with mechanisms based on electron transfer from the distal domain using the value suggested by the distance.
When similar considerations are applied to the E295 mutants, the naïve treatment of rate constant from the distal domain becomes more clearly flawed. An important technical point to note is that, because rates in the E295 mutants are so slow, they can be measured at a time, <50 ms, corresponding to that at which SQ is sampled. With \( t_{1/2} \sim 100 \) ms, most centers would still have heme \( b_L \) oxidized, so that an empirical rate constant for the bifurcated reaction could be determined directly. For the bulky sidechains (E295Q, E295W), the SQ would likely be constrained to the distal domain, and SQ occupancy would likely approach the maximal value allowed by the equilibrium constant for the first electron transfer, \( K_1 \), and by driving force. Measurement of occupancy for SQ in E295W therefore provides, for the first time, an empirical value for the rate constant of the bifurcated reaction. Using values measured at pH \( \sim 8.5 \), and by substitution in \( \nu = k_2([SQ.b_L\,^Ox]) \) we get a value of \( \sim 10^3 \, \text{s}^{-1} \), which is 3-orders of magnitude smaller than the value calculated from distance. We use approximate values ([SQ] in the range 0.05 – 0.1/QO-site (Fig. 3.1), and we use the rate \( \sim 60 \, \text{e}^\sim/\text{s}/\,bc_1 \) seen with E295Q (2), not \( \sim 15 \, \text{e}^\sim/\text{s}/\,bc_1 \) seen with E295W. This is because we recognize that occupancies might vary in different strains, and in E295W the bulk of the tryptophan might lead to artificial displacements in the site leading to lower rates than for more native side chains. As a consequence, this low rate constant calculated of \( \sim 10^3 \, \text{s}^{-1} \) is higher than if we had used rates derived from E295W data.

One clear conclusion is that the rate constant calculated from distance is inappropriate for the reaction under these conditions. Factors other than distance must come into play. Given the complexity of the second electron transfer step, it is perhaps not surprising that other parameters might modulate the rate. It will also be obvious that if the rate constant value \( \sim 10^3 \, \text{s}^{-1} \) from the distal domain is appropriate in WT, then this is not consistent with the concerted model. No wild type SQo occupancy consistent with the measured lag phases and the lack of any observable SQo could also be consistent with the observed uninhibited rate. Use of the same rate constant from the distal domain in wild type and E295 mutants is the most economical hypothesis, and is supported by our diffusion model of kinetics. These rates are also supported by the fact that bypass rates are similar in all strains, and were not acting to slow the apparent rate of electron transfer. While the rate constant calculated from distance is clearly inappropriate for transfer from the distal domain, if SQ were to move close to heme \( b_L \), the reaction might be a simple electron trans-
fer, and the value expected from distance (~10^9 s\(^{-1}\) for 6.5 Å) would then be appropriate, representing a maximal operational value. Movement of SQ to allow migration closer to heme \(b_L\), facilitated by rotational displacement of the E295 side chain, then becomes an attractive possibility (37).

3.2 Ultra-Fast Freeze Quenched EPR

Abstract

Experiments were performed on both the WT \(bc_1\) complex, and the \(bc_1\) complex that harbored a point mutation, E295Q. A semiquinone species with unique properties was observed across all five time courses, with some properties similar to those observed by Sarewicz et al (26), which they attributed to a SQ-ISPH state. Both Sarewicz et al. (26) and our spectra show power saturation properties consistent with a binding partner. However, while our spectra shows “wings” on either side of a central doublet, that of Sarewicz et al. (26) shows a single broad peak with smaller shoulders on either side. This indicates either a more complex binding state in our spectra, or may be due to the ability of ultra-fast freeze quenching to capture a higher range of conformers (see section 1.7).

As described in Materials and Methods, a disposable ultra-fast microfluidic freeze-quenching apparatus was developed for the purpose of analyzing the kinetics of SQ formation, the spectral properties in CW, and details of the reaction environment through pulsed EPR studies of neighboring protein and solvent nuclei. The bifurcated reaction at the \(Q_o\) site was initiated in the purified \(bc_1\) and inhibited by antimycin A to block the \(Q_r\)-site, in the fully oxidized and \(b_H\)-reduced complex. The fully reduced purified \(bc_1\) complex was initiated in the absence of antimycin A. Briefly: (i) by addition of excess cyt \(c\) to the \(b_H\)-reduced complex incubated in the presence of excess OH-decylUQH\(_2\), with \(b_H\) reduced and \(b_L\) oxidized at time=0; (ii) by addition of excess OH-decylUQH\(_2\) to the oxidized complex pre-incubated with excess cyt \(c\), with \(b_H\) and \(b_L\) both oxidized at time = 0; (iii) by addition of \(bc_1\) to a fully reduced quinone pool, followed by a dithionite titration to generate \(b_H\) and \(b_L\) reduced at t=0.
The kinetics of the formation of this SQ species were observed with time points over the range 166µs to 4ms. Comparison of the observed kinetics with kinetics simulated from a comprehensive model of the bifurcated reaction derived from parameters determined \textit{in situ}, showed agreement with overall trends across three initial redox states and in the E295Q mutant. Specific features were dependent on model parameters that would be expected to vary between our \textit{in vitro} system and the \textit{in situ} system from which the model was derived. Of particular interest are the previously unreported interactions between the SQ spin states and neighboring paramagnetic species, which are not yet fully understood.

**Experimental**

The general experimental approach was similar to that of Zhu et al. (3) as described in Materials and methods. The main difference was that samples of the isolated bc\textsubscript{1} complex for X-band CW EPR were collected under anaerobic conditions, and with both substrates (OH-decylUQH\textsubscript{2} and ferricyt \textit{c}) in excess to ensure that multiple turnovers could occur. Unless otherwise noted, reactions were performed in the presence of saturating antimycin A. Microfluidic ultra-fast freeze quenching was used to acquire samples at time points ranging from 166µs to 4.0ms. Under conditions in which both heme b\textsubscript{H} and heme b\textsubscript{L} were initially oxidized, the reaction was initiated by 1:1 mixing of OH-decylUQH\textsubscript{2}, 65µM, with the oxidized complex pre-incubated with excess equine ferricyt \textit{c} (800 µM). Under conditions in which heme b\textsubscript{H} was initially reduced and heme b\textsubscript{L} oxidized, the reaction was started by mixing 1:1 ferricyt \textit{c} (800 µM) to the complex pre-incubated with 65µM reduced OH-decylUQH\textsubscript{2}. Under conditions in which the complex was fully reduced, the reaction was initiated in the absence of antimycin A by 1:1 mixing of bc\textsubscript{1} pre-incubated with OH-decylUQH\textsubscript{2} and then subjected to a dithionite titration to fully reduce heme b\textsubscript{L}, with excess equine ferricyt \textit{c} (800 µM).

Time courses were collected using bc\textsubscript{1} complex from WT or from the E295Q mutant strain. In assays without pre-incubated 800µM ferricyt \textit{c}, the initial redox state of the hemes was monitored spectroscopically before sample collection. This was not possible with the bc\textsubscript{1} pre-incubated 800µM ferricyt \textit{c} since even the oxidized form of the cyt \textit{c} would saturate the detector at 800µM. Control spectra collected without pre-incubated ferricyt \textit{c} and without a reduced quinone pool confirmed an oxidized low potential chain under assay conditions. The OH-decylUQH\textsubscript{2} was maintained in a reduced state through catalysis using 70nM NDH-2 and 500µM
NADH. Concentrations of bc$_1$ monomer in the final mixed sample were between 1.8µM and 6µM, as determined by [cyt c$_1$], and in fitting all signals were scaled relative to [bc$_1$].

Ascochlorin at ~100µM was added to fully oxidized WT bc$_1$ complex solution in the presence of antimycin A immediately after the 3.5ms time point sample was collected as a negative control.

Concentrations of SQ$_o$ were assumed to change in proportion to the amplitude of the EPR spectrum, and the change in concentration over time was compared with that expected from an *in situ* kinetic model previously developed (2). The general behavior was well fit, using the published values for parameters. Only the relative ratios of bc$_1$ monomer and substrates were altered to bring the model more in line with ratios appropriate for our *in vitro* system. No attempt was made to use the Dynafit program to generate fits to the experimental data. As discussed in section 2.5, without a more thorough understanding of the quantum properties of our SQ$_o$ intermediate, its local environment and binding partners, without a more detailed study on the substrate binding parameters, and without modification of the model to include parameters describing the properties of the SQ$_o$ state, any optimization to our *in vitro* data using the current *in situ* model would be of questionable significance.

**Results**

The SQ$_o$ signal observed (see Fig 3.2) is unlike anything previously observed in Q$_o$ site SQ studies (4, 26, 41). However as discussed above, some features, in particular the possible interaction with ISPH, show similarity with properties observed by Sarewicz et al (26) which they attributed to a SQ-ISPH state. The kinetics of the SQ$_o$ species in this study were assayed with either the fully oxidized bc$_1$, the fully reduced bc$_1$ or the b$_{H}$ reduced bc$_1$ complex from WT, or the b$_{H}$ reduced bc$_1$ and the fully oxidized bc$_1$ complex from strain E295Q. The WT *in vitro* time courses were in agreement with the general trends simulated in our *in situ* derived model. The E295Q time courses were in agreement with the *in situ* model with k$_{diff}$ set to $10^2$ based on the work in (2) correlating observed turnover with theoretical diffusion rate. Varying EPR power and temperature also show results consistent with a coupled species, which we propose to be the ISPH, interacting through His-152. This hypothesis was further supported by subsequent pulsed
EPR analysis.

We observe the presence of two large peaks; one centered at the predicted \( g = 2.005 \), and the second peak at \( g = 2.029 \), with two smaller peripheral peaks centered at \( g = 2.036 \), and \( g = 1.98 \) (See Fig. 3.2). The relative intensities between these four peaks with respect to each other appear to remain constant across wide fluctuations in temperature from 5K to 77K; strongly indicating a single group of strongly coupled species, as discussed below. A broad peak centered at \( g = 2.06 \) is also present in this region, however it displays significantly different temperature dependence.

While the WT and E295Q CW spectra appear indistinguishable in shape, the differential temperature dependence of WT and point mutant E295Q complexes demonstrates some differences in their local SQ environments; thus pinpointing the source of the signal to the \( Q_o \) site. (See Fig 3.8).

In the presence of antimycin A, the fully oxidized WT \( bc_1 \) complex sample at 3.5ms showed a 49% drop in signal intensity after the addition of ascochlorin. This is consistent with the results observed by Berry et al. 2010 (42) where in the presence of antimycin, ascochlorin was seen to inhibit half of the \( Q_o \) sites.

**Discussion**

**Occupancy Values.** The SQ species observed here has properties unlike any previously seen, and the coupling to neighboring paramagnetic centers means that spin counting through internal standards is not sufficient to determine occupancy. Any unambiguous determination of occupancy will require more detailed understanding of spin-coupled partners, and quantum mechanical parameters of such partners including distances and spin state(s), and coupling. Attempts to identify binding partners through HYSCORE failed to yield plots with sufficient signal to noise to justify clear conclusions. However, as discussed in detail below, ESEEM spectra are very consistent with coupling through the pyrrole nitrogen of a histidine. The most likely candidate is \( N_e \) of His152, a direct ligand to Fe2 of the ISC, and likely H-donor in the H-bond stabilizing the \( ES \)-complex.

In collaboration with the Schulten group, we have recently published a new model for the reaction complex based on MD simulation of the \( ES \)-complex, and quantum chemical simulation of atomistic details (Barragan et al. 2015). Further developments using the most plausible quan-
tum chemical model as a starting point, involve prediction of the reaction trajectory through calculation of an implicit reaction coordinate. Results so far provide insights into the reaction pathway consistent with the general scheme suggested by our Marcus-Bronsted model (Ilia Solov'yov, personal communication). The trajectory of the reaction leads to a product state that might be similar to our SQ₀ state. These approaches suggest a possible pathway towards a more complete model that might provide the information needed for calculation of occupancy.

A large increase in signal intensity relative to the $S=\frac{1}{2}$ SQ₀ signal seen in (2) and in figure 3.1 is consistent with the coupling of the SQ₀ with the ISC. In addition, while in need of refinement, our model predicts a significantly higher occupancy levels than observed in (2), even without including potential stabilizing effects of a SQ-ISPH interaction.

Quantum-based models of SQ₀ with its binding partners will allow us to test the feasibility of possible SQ₀ interactions by attempting to reproduce of our experimental X-band CW EPR signal with its four peaks in a simulation. Coupling these simulations with direct structural data through pulsed EPR would allow for still further testing of hypothesized interactions. Interactions observed in EPR and tested in EPR simulations could then be incorporated into kinetic models, which would allow signal intensity to then be reliably correlated to occupancy. A fully parameterized kinetic model of the $bc_1Q_0$ site is an ongoing project in Crofts lab (Stuart Rose, personal communication), and further development will focus on better defining the gating and control mechanisms by incorporating the interactions suggested by our SQ₀ state.

**Time courses** The observed trends in the kinetics of SQ₀ formation are in agreement with the trends simulated from our diffusion-based model of WT $bc_1$ across multiple initial redox states. The observed trends in the kinetics of SQ₀ formation in E295Q are in agreement with the trends simulated from our diffusion-based model of E295Q $bc_1$ across multiple initial redox states. The E295Q mutation was reflected in the model as a $10^5$-fold lower $k_{\text{diff}}$ as predicted in Victoria et al 2013(2). Global simultaneous comparisons between all WT simulations and WT experimental data continues to show consistency in overall trends, however discrepancies in specific values of intensity highlights the need for model refinement from an *in situ* to an *in vitro*
system (Fig 3.4).

Figure 3.3 shows simulated WT SQ\textsubscript{o} formation kinetics, and our observed signal intensity of SQ\textsubscript{o} formation plotted over the first 4 ms. The kinetic trends of the simulated data shows agreement with the experimental data. All experimental intensity values were scaled in proportion to experimental [bc\textsubscript{1}], and scaled to account for instrumental deviation between each time course. As predicted, the observed formation kinetics of SQ\textsubscript{o} are dependent on the initial redox state of the low potential chain hemes. While there is an oxidized heme in the low potential chain, any SQ\textsubscript{o} intermediate formed will very quickly be oxidized to quinone, and the SQ\textsubscript{o} occupancy levels will remain undetectable. In WT bc\textsubscript{1} once the low potential chain is fully reduced, any SQ\textsubscript{o} intermediate formed will have nowhere to deposit its second electron, and the SQ\textsubscript{o} will accumulate. As we observe in figure 3.3, the WT bc\textsubscript{1} complex with a fully reduced low potential chain reaches a maximum [SQ\textsubscript{o}] within the first 1 ms. This indicates SQ\textsubscript{o} forming during the very first Q\textsubscript{o} site turnover, which has a rate of \(~1/\text{ms}\). For the fully oxidized WT complex, we see a lag phase with significant signal not forming until after 3ms. This indicates SQ\textsubscript{o} forming within the third turnover, since it first requires two turnovers to fully reduce the low potential chain hemes. The WT bc\textsubscript{1} with only b\textsubscript{H} initially reduced shows kinetics in between these two.

Figure 3.4 shows global simulated WT SQ\textsubscript{o} formation kinetics of all three redox states plotted against the observed signal intensities. While the general trends agree between the experimental and the simulated datasets, there is clear discrepancy between the relative intensity values between the simulated and experimental data. As discussed below, this discrepancy between relative intensities and agreement between trends is expected when comparing our in vitro data with our in situ model, where the most parameters we expect to be most affected are the binding rate of substrates.

Figure 3.5 shows simulated E295Q SQ\textsubscript{o} formation kinetics and our observed signal intensity of SQ\textsubscript{o} formation over the first 4 ms. As shown in Victoria et al 2013 (2), the diffusion model of the Q\textsubscript{o} site predicts a \(k_{\text{diff}}\) of \(~10^{2}\) for the E295Q mutant. This was the sole parameter altered between WT and E295Q models after ratios between enzyme and substrate were matched to exper-
imental conditions. The kinetic trends of the simulated data shows agreement with the experimental data.

Under the diffusion-based Q₀ site model, the theorized 10²-fold lower k_{diff} of E295Q (2) prevents the SQ₀ from being immediately oxidized by the low potential hemes. The SQ₀ first accumulates in the distal domain before diffusing towards the bₗ heme where the SQ₀ is oxidized. As in the WT bc₁ complex, we do see a difference in kinetic trends depending on the initial redox state of the bc₁, however, as predicted by our diffusion-based model, this affect of the initial redox state is far less pronounced in E295Q than in WT. The most dramatic difference being between the E295Q and WT fully oxidized kinetics, with a 2-3ms lag phase in WT and no distinct lag in E295Q. The appearance of a small lag in the first 0.5ms of the b_H-reduced E295Q is not understood and may be attributed to experimental error. However, the overall trends observed with b_H initially reduced are in agreement with simulations, where we see E295Q plateau within the first millisecond, while the WT takes more than twice that long (Fig 3.6).

Simulation Parameters. Correlating the observed turnover kinetics of E295Q published in (2) with the predicted rate constant of diffusion for similar turnover kinetics in the diffusional model (2), gives us a k_{diff} of ~10² predicted for E295Q, versus 10⁷ for the WT bc₁. The kinetic models for turnover within the Q₀ site of a bc₁ complex with an implicit inhibitor in the Qᵢ site are as described materials and methods, and in Victoria et al. (2) for bₗ(ox)b_H(red), bₗ(red)b_H(red), and bₗ(ox)b_H(ox) complexes at time = 0.

The published models were originally parameterized using data from in situ chromatophore systems, therefore as we apply it to an in vitro system with purified bc₁ some parameters are called into question. Specifically; i) our acceptor cytochrome is equine cyt c, not R. sphaeroides cyt c2, with a potential difference in rate of reduction by cyt c₁ and in binding affinity; k₃ and k₋₃. ii) Differences in the H-bonding network within the Q₀ site between WT and E295Q bc₁ also has the potential to also affect k₃ and k₋₃ values between strains as described in Devanathan et al. (63) iii) while Victoria et al. (2) demonstrated little change in the Km of QH₂ between E295 mutants, we must allow for differences in the rate constants while keeping the ratio between the forward and reverse reactions fixed. iv) differential apparent QH₂ binding kinetics between the
oxidized and reduced samples may be expected due to pre-binding versus non pre-binding methodologies.

**CW X-band Power Saturation and Temperature Sensitivity.** Some properties of the SQ₀ species observed in these experiments are surprising, because they differ in several important respects from those previously observed. The line shape shows hyperfine “wings” not seen in previous mix-hold work, indicating either a modified molecular orbital, or perhaps spin interaction with a neighboring paramagnetic species, which both mechanism theory and ESEEM data (see below) suggest to be a histidine pyrrole nitrogen. Additionally, the power saturation and temperature dependence also differ from previous publications. The signal saturates only at much higher power, indicating, in support of the above, faster relaxation through spin coupling. We also see differences between the patterns of WT and E295Q temperature dependence on signal, though both deviate similarly from Curie law; consistent with spin coupled systems and small changes in electrostatic environment in the mutant.

**Power saturation.** This is performed from 0.2mW to 200mW at multiple temperatures for the 0.25ms WT freeze-quenched sample, and at 77K for the 1ms E295Q sample. As shown in Fig. 3.7, when compared to previously published power saturation curves of the 10ms sample of Cape et al.(4) in the presence of antimycin, (Fig. 3.7 inset, filled squares) we see a distinct shift in the intensity required for saturation. This increase in P₁/₂ is consistent with spin interaction of the semiquinone species with another spin. For WT, signal amplitude was determined as a function of microwave power at three temperatures; 5K, 15K, and 70K. For E295Q, signal amplitude was determined as a function of microwave power at 77K. Saturation onset occurred at lower power at 5 K than at 15 K, both peaking at √P ~4, and peaked at higher power at 70 K and 77K for E295Q (√P ≥8). However, saturation at all temperatures was much less marked than that observed in previous work. The insert shows for comparison the saturation characteristics of the SQ₀ species detected in earlier experiments (4) peaking at √P ~1.

**Temperature Sensitivity.** As shown in Fig 3.8, CW X-band EPR was performed side by side on the WT and E295Q 250µs samples for b₁(red)b₁(ox) at time = 0. Spectra were collected at multiple temperature values. If both follow the Curie law for a single uncoupled spin, then
plotting the log of signal intensity versus 1/T, you would expect parallel straight lines with a constant vertical shift between them. Curie law is dependent on the number of spins and the magnetic moment; assuming non-saturating conditions with no temp dependent effects such as multi-conformers, and that it’s a non-coupled system. As we can clearly see, both traces are curved, with significant difference in the degree of temperature sensitivity between WT and E295Q bc1 (60, 61)

As seen in temperature dependent CW as well as ESE spectra (Figs 3.9, 3.10), a prominent peak around g=2.06 becomes obvious as the temperature of measurement is lowered from 70 K to 5 K, is detected as a fast decaying component at 32 K. This same component is obvious in the CW spectrum at lower temperatures, and is barely detectable at 60 K (Fig 3.10).

**Discussion**

One of the key sources of controversy over the last several decades on the mechanism of the Qo site redox reactions has been the inability to directly monitor the Qo site semiquinone intermediate, leading to a wide proliferation of debated models (35, 49). In recent years, multiple groups have been able to isolate the Qo site SQ species (2, 4, 26, 28), however on timescales that were orders of magnitude beyond what would be necessary to monitor its initial formation. Zhu et al. (3) was masterfully able to monitor for the SQo signal on the sub-millisecond timescales necessary to observe SQ formation. However, as discussed above, their experimental conditions provided only a single turnover, and with an oxidized low potential chain, the SQ requires multiple turnovers to appear. Building on these previous studies, our system has allowed us to observe initial formation kinetics of a SQo signal for the first time, which we have determined for three different initial redox states in WT and Qo-site mutant E295Q. Within constraints discussed above, the observed kinetics follow the pattern suggested by the diffusional model. However, the spectroscopic properties of the SQo species detected are quite different from those reported previously.

The CW spectra of the SQo state obtained with ultra-fast freeze quenching shows a complexity of peaks, quite different from the spectra observed in previous publications, all of which were obtained at 10ms and longer after mixing, and with mix-hold cryobath freeze quenching (4, 28). In contrast to the single symmetrical peak suggesting a simple S=1/2 uncoupled spin system, the
four peaks of our spectra show a complex spin state or combination of states. The amplitude of the signal detected is also substantially higher than expected from our previous studies observing a simple \( S=\frac{1}{2} \) SQ signal. Because the signal intensity of CW EPR increases exponentially with the spin state of the radical, proportional to \( S[S+1] \) (Weil, Bolton 2007(62)), the high intensity of this SQₙ signal could be explained by spin-sharing with the coupled partner. However, the range over which the additional peaks are observed is much narrower than the normal ISPH signal, suggesting spin transfer to the SQ rather than a simple sharing of spins between the partners. The difference in properties between this and previously published signals may indicate that, by rapid freezing at lower temperature, we have trapped the semiquinone in an early EP-complex state, from which the SQ had dissociated under the trapping conditions of earlier experiments.

**Spin Coupling Partner.** In the only previous work to have used high-resolution approaches, ENDOR and ESEEM spectra showed no evidence of H-bonding partners, and no spin interaction with neighboring N-atoms (4). Nevertheless, we propose a high spin coupled species formed from an iron of the 2Fe2S complex and the newly-formed SQ through an H-bond with the \( N_e \) of His152. Possible candidates for paramagnetic partners in the Q₀ site are the reduced ISPH, and the ferriheme \( b_l \). In the light of the reaction trajectory expected from the kinetic model, and the narrow grouping of bands around \( g \sim 2 \), we can say that spin coupling with a ferriheme group is highly unlikely. Under conditions in which heme \( b_l(\text{ox}) \) is present in our model, any SQ₀.ISPH• complex formed in the native complex is expected to be short lived because the SQₙ would rapidly dissociate to facilitate electron transfer to the heme, and the ISP would then rapidly diffuse from the cyt \( b \) binding domain to carry its electron to cyt \( c_1 \). The complex between the SQ₀ and the ISPH under metabolically backed-up conditions (with heme \( b_L \) reduced) is clearly stable at liq. N\(_2\) temperatures on the 10 ms scale, but sufficiently unstable as to dissociate at higher temperature and/or longer times.

Due to the presence of excess oxidized cyt \( c \) as acceptor, heme \( c_1 \) is expected to be oxidized, and the cyt \( c \) acceptor itself predominantly oxidized, but both hemes are likely quite distant from the SQ₀. Even if coupled to the SQ, heme species have spectra very far removed from the \( g=2 \) region of the SQ. Since a coupled spectrum is a hybrid of the spectra of its participating groups, it is difficult to envision a heme-SQ coupled spectra remaining centered at \( \sim g=2.0 \). The reduced
ISPH, however, has peaks centered at \( g = 2.02, \ g = 1.89, \) and \( g = 1.80; \) all centered a few hundred gauss around the \( g = 2.005 \) SQ peak, though with much wider band width.

In the light of the observation of this stable coupled state at very low time scales, it is necessary to consider the presence of a \( Q_o \) site gating mechanism where, under conditions with a blocked low potential chain, the reduced ISPH does not dissociate, but remains bound to cyt \( b \). This bound state coupling with the \( SQ_o \) would thus help to prevent harmful ROS formation in bypass reactions. Our model currently only relies on electrostatic repulsion between reduced \( b_L \) and the anionic \( SQ_o \) to avoid bypass reactions. However, a bypass prevention mechanism through ISP bonding had been previously proposed by Link et al (40) where he suggests that the reduced ISP has several orders of magnitude higher affinity for \( SQ_o \), though this was proposed in the context of an immobile \( SQ_o \).

**Power Saturation and Temperature Sensitivity**

**Power Saturation.** Cape et al. (4) noted that the failure to see an increase in power level required to saturate their \( SQ_o \) signal might indicate no “strong coupling of the SQ EPR signal to paramagnetic centers, such as reduced 2Fe2S”. Since we see these effects, the strong coupling provides an explanation for the behavior seen here. Cape et al (4) also offer as an explanation for this lack of interaction the possible migration of the \( SQ_o \) into the proximal niche of the \( Q_o \)-site; further away from the ISC. E295Q shares this same saturation point and midpoint as WT at 70K.

**Temperature Sensitivity.** While the identity of the observed peak with differing temperature dependence to the \( SQ_o \) is unknown, it appears to consistently appear in proportion to the \( SQ_o \) signal, and it appears at \( g = 2.06 \) in proximity to the 2.02 g-value of the ISC. Additionally, in the simulations of Sarewicz et al.(26) of \( SQ_o \) coupled to the ISC, this \( g = 2.02 \) ISC peak is the only one remaining after coupling with the \( SQ_o \). In addition, as discussed below, ESEEM spectra taken at this \( g = 2.06 \) feature have the same characteristics as the \( SQ_o \) ESEEM spectra taken at \( g = 2.0054 \). These facts all lead us to speculate that this \( g = 2.06 \) peak is a component of the ISC, though the differing temperature dependence is surprising.
As shown in Fig. 3.8, plotting the log of scaled signal intensity with respect to 1/T over changing temperatures gives a clear deviation from Curie law that would predict straight lines. Curie Law, however, depends on an uncoupled system; adding support to the hypothesis of a SQ₀ coupled with the ISC. In addition, the clear difference in the degree of temperature sensitivity highlights differences between the environments of the WT and E295Q SQ₀ signals; pinpointing the source of the signal to the Q₀ site.
Fig. 3.1 Direct measurement of rate constant \( (k_2) \) for the 2\textsuperscript{nd} electron transfer of the bifurcated reaction when \( \text{SQ}_o \) is constrained to the domain of the \( Q_o \)-site distal from heme \( b_L \). In the E295W strain, the \( \text{SQ}_o \) is constrained to the distal domain. The kinetics of \( QH_2 \) oxidation can be measured from cytochrome changes with \( \text{SQ}_o \) maximally populated, and the acceptor (heme \( b_L \)) oxidized. \( \text{SQ}_o \) occupancy was assayed in WT and E295W strains on mixing oxidized \( bc_1 \) (with excess cyt \( c_\alpha \)) with followed by liq. propane freeze-quench at \(~30\text{ms}\) (see Victoria et al.)
(2). Observed $b_{H}$ reduction rates were $\sim 60 \text{e/s} \ bc_{1}$ for most E295 mutants, and $\sim 20 \text{e/s} \ bc_{1}$ for E295W, compared with $\sim 1000 \text{ s}^{-1}$ for wild type,

![CW EPR traces of the $b_{H}$-reduced WT $bc_{1}$ collected between 0-4 ms, Intensities are arbitrarily offset for viewing. The shape of these spectra are representative of all four time courses across WT and E295Q.](image)

Fig. 3.2 **CW EPR traces** of the $b_{H}$-reduced WT $bc_{1}$ collected between 0-4 ms, Intensities are arbitrarily offset for viewing. The shape of these spectra are representative of all four time courses across WT and E295Q.
Fig. 3.3 (cont. on next page)
Fig. 3.3 (cont. on next page)
3.3 Wild type kinetics. Simulated kinetic data (A,C,E) and observed SQ$_o$ EPR signal (B, D, F) in three initial redox states (i) $bc_1$ fully reduced (A,B), (ii) $bc_1$ fully oxidized (C,D), and (iii) $b_H$ of $bc_1$ reduced (E,F).
3.4 Global comparison. Simulated kinetic data plotted against observed freeze-quenched samples. Simulated fully oxidized (blue line), simulated $b_H$ reduced (red line), and simulated fully reduced (green line). Experimental fully oxidized (orange circles), experimental $b_H$ reduced (blue boxes), and experimental fully reduced (purple triangles).
Fig. 3.5 (cont. on next page)
3.5 E295Q Simulated kinetic data plotted against observed freeze-quenched samples with $bc_1$ fully oxidized (A,B), and $b_H$ reduced (C,D). There is a break in the simulation of the fully oxidized complex due to a difference in $bc_1$ concentration used in 0.38 and 0.78ms relative to 0.16 and 1.53ms.
Fig. 3.6 E295Q WT comparisons. Red squares: WT experimental. WT experimental (blue diamonds), and E295Q experimental (red squares). Initially fully oxidized complex (top), initially $b_{11}$-reduced complex (bottom).
Fig. 3.7 Power saturation of WT $bc_1$ SQ$_0$ at multiple temperature values, and E295Q SQ$_0$ at 77K, which shares the same saturation point and midpoint as WT at 70K. Inset; power saturation as observed by Cape et al. (4), where a low power saturation is indicative of a lack of interactions with SQ$_0$. 
**Fig. 3.8** Singal intensity scaled to intensity at 10K; E295Q (blue diamonds) ant WT (red squares). Both are 250μs samples from $b_{H}(\text{red})b_{L}(\text{ox})$ at time=0. Temperature sensitivity demonstrating deviation from curie law.
3.9 CW X-band EPR of the 4ms WT(bH reduced at time =0) sample over a temperature gradient of 5K to 32K. Signal intensities are scaled as ESE relative signal intensities are not quantifiably significant. The peak emerging at lower temperatures is centered at $g = 2.06$. Inset is CW spectrum taken at 5K.
3.10 CW X-band EPR of the 250µs WT(b_H reduced at time =0) sample over a temperature gradient of 5K to 60K. The peak emerging at lower temperatures is centered at g=2.06.
Chapter IV Ultra-Fast Freeze Quenched EPR; pulsed ESEEM

Abstract

In an effort to better identify the group or groups coupling with the SQ₀ intermediate that we hypothesize to be part of a bypass control mechanism, pulsed EPR ESEEM has been conducted. ESEEM has allowed us to look for spins interacting with the SQ₀, and then to correlate the resulting spectra with known characteristics of candidates for SQ₀ interaction. ESEEM of multiple peaks within the SQ₀ signature g values shows a low frequency triplet in the nitrogen region; characteristic of interaction with a pyrrole nitrogen of a histidine ring. This result is consistent with our knowledge of possible paramagnetic binding partners as discussed above.

Experimental

As discussed in Materials and Methods for ESEEM, Values of τ were scanned iteratively to obtain τ values with optimal signal:noise before data collection over a larger average. WT ESEEM spectra were measured at 120ns, 15K; 010615 4ms. 120ns decayed sample was measured at 35K. E295Q ESEEM plot was measured at a tau value of 136ns at 29K.

Results

As shown in figure 4.1, ESEEM data across multiple τ values from wild type b₅₆(red)b₅₇(ox) at time = 0. freeze quenched SQ₀. In contrast with the data from Cape et al (4), strong peaks are seen in the nitrogen interaction region between 0-3 MHz Spectra are taken at the SQ peak centered at g=2.005 and at the broad peak centered at g=2.06 which has different power and temperature saturation characteristics. Small differences are observed in frequencies, most notably the higher amplitude of the peak at ~1.7 MHz from the g=2.06 line, and the broader peak at ~0.8 MHz from the g=2.005. The differential amplitudes, and the difference in decay with temperature suggest that the spectra come from different paramagnetic species. However, the similar patterns in peak frequency along with similar periodic responses to changing τ values, suggest an interacting, though not directly spin coupled, species. (Fig. 4.1) shows us ESEEM taken at g values of 2.006 and 2.029, which are the central twin peaks of the SQ₀ spectrum, while g=2.06 rep-
represents the broad side peak with a differing temperature dependence but similar ESEEM spectra. The decayed 2.006 signal represents a sample which has decayed over time and lost the CW peaks centered at $g$ values of 2.029 and ~1.99. All spectra except for the “decayed” sample share the same triplet profile of three peaks centered at 0.4, 0.9, and 1.7 MHz

Three-pulse ESEEM spectra collected at different field positions of the EPR spectrum (Fig. 4.1) show a peak at $^1$H Zeeman frequency ~14. 7 MHz resulted from the interaction with protons in the environment of the electron spin and the three peaks; 0.4, ~0.9, and 1.7MHz in low frequencies region appropriate for two $^{14}$N transitions. Observation of the values of these two frequencies allows us to suggest the interaction with the protonated nitrogen from the imidazole ring of histidine. They can be assigned to the nuclear quadrupole frequencies of imidazole nitrogen (47, 57)

$$\nu_0 = 2K\eta, \quad \nu_- = K(3 - \eta), \quad \nu_+ = K(3 + \eta)$$

with

$$\nu_+ = 1.7 \text{ MHz, and } \nu_- = \nu_0 = 0.8-0.9 \text{ MHz}$$

that defines quadrupole coupling constant $K = e^2qQ/4\hbar ~0.425 \text{ MHz}$ and asymmetry parameter $\eta ~1$. These values are very typical for the protonated imidazole pyrrole nitrogens observed in different systems including metal-histidine complexes (41) and semiquinone hydrogen bonded with a D or L histidine, imidazole, or urocanate (47).

**Discussion**

The ESEEM spectra for the SQ$_o$ species as well as on the side peak centered at $g=2.06$ are consistent with SQ$_o$ interaction with the pyrrole nitrogen of His-152 as suggested by the results of CW EPR, structural theory, kinetic SQ$_o$ formation, and CW signal saturation. The similar ESEEM spectra observed at $g=2.06$ and $g=2.0054$ suggest these peaks come from interacting species, however the differing temperature dependence indicates that the signal at $g=2.06$ does not likely come from a directly coupled species.
Deligiannakis et al (57) observed a highly similar low frequency triplet with peaks at ~0.4, 1.5, and 2.5MHz in their photosystem II QA-plastoquinone radical studies, where the QA-radical was observed through in-depth 3-pulse ESEEM with and without isotopic labeling of the photosystem. In this study, the imino nitrogen of His215 is discussed as a candidate for the source of the nitrogen interactions with QA, while subsequent crystal structures providing resolution to 1.9Å Umena et al. (59) revealed a clear nitrogen bond from His 214, with His 215 bonding with QB. The ESEEM signal from this system, especially their low-frequency triplet is of special interest as the crystal structures of PSII reveal an H-bond network connecting the plastoquinone radicals with histidines hat are bonded to an iron group Ishikita et al. (58). This is clearly analogous to our proposal of a SQ to H152 to 2FeS hydrogen bonding network.

In a study by Tryshkin et al. (65) their goal was to look for patterns in ESEEM spectra for radical to pyrrole nitrogen interactions. They performed a thorough study of hemoglobin (Hb) and Hb subunits in a variety of conformations as well as model complexes with imidazole; all interacting with NO radical. The fact that this is a NO radical, and not a SQ radical should not affect the spectrum since in principle quadrupole coupling of a radical with a pyrrole is defined primarily by the pyrrole group itself (Dr. Dikanov, communication (60)). In this radical-pyrrole ESEEM study, a low frequency triplet is seen with peaks at 0.33, 1.18, and 1.51, demonstrating the relation of additivity (65, 60), again indicating three peaks in the low frequency nitrogen region with three transitions; 0 to 1, 1 to 2, and 0 to 2, where the frequency of the third transition is equal to the sum of the first two; consistent with a pyrrole nitrogen.

While our frequency values of 0.4, 0.9, and 1.7 MHz do deviate from perfect cancellation conditions, the slight nature of this deviation allows for calculation of a rough estimate for values of η and K, yielding ~1, and 0.425 MHz, respectively; consistent with values published for pyrrole nitrogens of histidine (47).
Fig. 4.1 (cont. on next page)
Fig. 4.1 (cont. on next page)

0.4, 0.9, 1.7 MHz: $^{14}$N triplet

~14.5 MHz: $^1$H singlet
Fig. 4.1 ESEEM collected at $g = 2.06$ (A, D; side and top views), and $g = 2.0054$ (B, C; side and top views); 15K, 4ms sample of WT $b_L$(red)$b_L$(ox) at time = 0. Both proton coupling and nitrogen coupling are observed, with the low frequency triplet being consistent with interaction with a pyrrole nitrogen.
References


11) World Malaria Report 2014; World Health organization


29) Dr. Alex Taguchi, personal communication


31) A. R. Crofts, V. P. Shinkarev, S. A. Dikanov, R. I. Samoilova, D. Kolling, Interactions of quinone with the iron-sulfur protein of the bc(1) complex: is the mechanism spring-loaded? *Biochim Biophys Acta.* **1555** (2002).


60) Dr. Sergei Dikanov; communications

61) Dr. Mark Nilges; communication


