IDENTIFYING THE MECHANISM OF SECOND PROTON RELEASE FROM THE $BC_1$ COMPLEX $Q_0$-SITE

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biophysics and Computational Biology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2017

Urbana, Illinois

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Abstract

The cytochrome $bc_1$ complex and its family members play a central role in biological energy transduction across all domains of life, oxidizing $\text{QH}_2$ in a bifurcated reaction which generates a proton-motive force used for ATP synthesis. It is widely accepted to operate according to a modified Q-cycle, but there is still debate over some aspects of the mechanism. We used the pH-sensitive dye neutral red to study the release of protons from the $bc_1$ complex, with specific focus of the mechanism for release of the second proton from the $Q_0$-site. Partial processes for the proton release could be isolated through use of different inhibitors, and correlated with electron transfers through the complex. We tested mutations at residues R94, N279, and Y147 in the $b$ subunit of the complex, which are thought to interact with the water chain which provides a pathway for release of the second proton. Mutants R94A, N279F, and Y147T showed a significant decrease in proton release for a second turnover compared to the first, an indicator that the proton from the first turnover had difficulty leaving the complex. The latter two mutants also showed an intriguing difference in kinetics for the proton release and electron transfers for the first turnover.
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Chapter 1: Introduction

1.1 Purpose of study

While there is good evidence establishing the identity of the primary proton acceptor(s) for the second proton, there is still lively debate over the point, and the subsequent path for proton release is even less well-established. The main goal of this study is to provide insight into the mechanism and pathway for the second proton release from the $Q_o$-site.

1.2 Structure of the $bc_1$ complex

The conserved core of the $bc_1$ complex is a multi-subunit homodimer [1]. Members of the family of $bc_1$ complexes are found in respiratory and photosynthetic systems across all domains of life. Their main function is to use redox energy from respiration or photosynthesis to generate both the electrical and chemical components of the proton gradient used for ATP synthesis. Despite billions of years of evolution since the last common ancestor, the structure of the catalytic core and the mechanism of enzyme activity are highly conserved across all domains of life. Thus, studying the $bc_1$ complex in a photosynthetic bacterium, *Rhodobacter sphaeroides*, provides insight into the function of this mitochondrial machine, important in medicine, agronomy, and the drug industry, because of roles in generation of reactive oxygen species (ROS), and as a target for fungicides, pesticides and drugs.

The quinol oxidation site ($Q_o$-site) of the enzyme, located on the P-side of the membrane (side with positive proton potential), oxidizes quinol (QH$_2$) to quinone (Q) in a bifurcated reaction. For each QH$_2$ oxidized, one electron passes down the high-potential chain (HPC) and reduces a mobile cytochrome $c$ on the same side of the membrane as the $Q_o$-site, while the other electron crosses the membrane to the enzyme’s quinol reduction site ($Q_i$-site) via the low-potential chain (LPC). The two protons are released on the P-side of the membrane, through different pathways. The paths taken by the first proton and both electrons have long been identified, although there are
still questions regarding the details of the transfer mechanisms. One monomer of the \(bc_1\) complex is shown in Figure 1.1. The \(b\) subunit contains the \(Q_o\)- and \(Q_i\)-sites, and the two \(b\) hemes of the low-potential chain (\(b_L\) and \(b_H\)). The Rieske iron-sulfur protein (ISP) contains the first component of the high-potential chain, a 2Fe-2S cluster, on a mobile domain that is able to dock with either the \(b\) subunit at the \(Q_o\)-site or with the \(c_1\) subunit. The \(c_1\) subunit contains a \(c\) heme that serves as an intermediary in the high-potential chain between the 2Fe-2S cluster and a soluble electron carrier, cytochrome \(c_2\). There is a water-filled cavity adjacent to the \(Q_o\)-site and heme \(b_L\), which is thought to contain the exit pathway for the second proton released during \(Q_o\)-site turnover.

1.3 The Q-cycle

The \(bc_1\) complex is understood to operate according to the modified Q-cycle [2] (Figure 1.1). The overall reaction is given in eq. 1.1.

\[
\text{QH}_2 + 2 \text{ferricyt c} + 2\text{H}_N^+ \rightleftharpoons \text{Q} + 2 \text{ferrocyt c} + 2\text{H}_P^+ + 2\text{H}^+_{\text{scalar}} \quad \text{eq. 1.1}
\]

Quinol binds in the distal pocket of the \(Q_o\)-site (farther away from heme \(b_L\)). It forms a strong hydrogen bond with a histidine ligand of the cluster (H152 in \textit{Rb. sphaeroides}) of the oxidized ISP, which stabilizes the \(ES\)-complex. A second weak H-bond may form with E295 or Y147 in subunit \(b\). The rate-limiting step for the turnover of the \(Q_o\)-site is the reduction of ISP by \(QH_2\), and involves transfer of both an electron and a proton through the H-bond with H152 to generate (initially) the neutral SQ (\(QH\)), the reduced cluster, and an associated protonation of H152. The mobile head domain of the now-reduced iron-sulfur protein, generally shown as ISPH, rotates and binds to the cytochrome \(c_1\) subunit. ISPH reduces cyt \(c_1\), releasing the first (scalar) proton into the P-phase. Cyt \(c_1\) is oxidized by a mobile electron carrier, cyt \(c_2\). The coordinated transfer of electron and proton through a common path has important mechanistic consequences.

The second proton and electron transfers occur through separate pathways. The proton from \(QH\) is passed to a proton exit pathway including E295 as acceptor, but recent MD-QM
studies have suggested that transfer may involve Y147 as an intermediate H-bonding group, and a more complex process than simple transfer along the hydrogen bond suggested in earlier models. E295 (and Y147) are thought to rotate to interact with a common group of water molecules stabilize by H-bonding to residues including N279, R94 and the heme $b_L$ propionates with which it H-bonds. This group of waters extends to a proton-transferring water chain leading to the exterior P-phase (aqueous phase with positive proton potential). Examining the role of these residues in facilitating proton release is the object of this study. Rotational displacements of the sidechains of E295 and Y147 open a volume close to heme $b_L$, allowing the semiquinone to move close enough to transfer the electron at the rates observed. At some point, the proton is released to the water chain. If this happens early in the sequence (the most likely scenario), the semiquinone anion, $Q^-$, migrates in the active site. If later, it will be the neutral form, $QH$. On electron transfer, the electron travels down the low-potential chain to heme $b_L$, then heme $b_H$, and finally to the $Q_i$-site on the other side of the membrane. The separation of proton and electron, and transfer along different pathways, necessarily has coulombic consequences, which have engendered much discussion centered on roles in control of the reaction, especially in the context of a regulation to minimize generation of damaging ROS. Dissection of these pathways may therefore allow a better understanding of these more esoteric aspects, with possible applications in medicine.

A second turnover of the $Q_o$-site is necessary to fully reduce the quinone at the $Q_i$-site, returning the enzyme to its original state and regenerating one $QH_2$ in the process, but introduces complications due to overlap of effects from first and second turnovers. These difficulties can be minimized by judicious use of inhibitors, allowing development of experimental protocols to facilitate deconvolution of the separate contributions. In addition, the recent development of a comprehensive kinetic model has helped to clarify the reaction profile.
1.4. Model organism

*Rb. sphaeroides* and related species have seen extensive use as model organisms for studying the *bc*₁ complex. *Rb. sphaeroides* uses the *bc*₁ complex both during aerobic respiration and during anaerobic photosynthesis. The protein can be purified, or the entire photosynthetic chain can be studied in membrane vesicles, the chromatophores (see below), in which the proton pumping activities can be studied, providing many avenues for experimentation. Many structures of mitochondrial and bacterial complexes with resolution better than 2.5 Å have been published [3-5], and provide a framework for detailed discussion of mechanism. These structures have also been used as the basis for computational simulations using molecular dynamics and quantum chemical approaches.

When grown photosynthetically, *Rb. sphaeroides* forms sausage-like invaginations of the bacterial inner membrane which contains the photosynthetic apparatus. On mechanical disruption, these invaginations break up to form vesicles, the chromatophores [6]. These contain a complete apparatus for the light-driven reaction [7], including 12-20 reaction centers driving the photochemical reaction (oxidation of ferrocyt *c*₂ and reduction of ubiquinone), 6-10 *bc*₁ complexes (which catalyze the reverse process, as in *eq. 1*), light harvesting complexes LH1 and LH2, and additional ancillary membrane complexes at lower stoichiometry. On activation by a saturating short (~5 μs) flash, kinetics can be followed through the absorbance changes, which are dominated by turnover of the cytochromes of the photosynthetic chain, including the *bc*₁ complex. In the mean, the reaction centers generate the substrates for the *bc*₁ complex, and under normal conditions of growth, the stoichiometry of the components leads to a complete cycle returning to the system to the initial state in most chains. Under these conditions, the *Q*₀-site of each *bc*₁ complex turns over twice to release 4 H⁺ to the internal *P*-phase as shown in the overall reaction above [1, 8]. Ideally, this would require 2RC:1*bc*₁ complex:1cyt *c*₂, but heterogeneity in distribution of the
centers [9] means that although the observed behavior is close to this ideal, it hides a statistical range centered around these values. Under different conditions of growth, the mean can vary widely from this ideal, and interpretation of experimental outcome has to take account of these factors.

### 1.5 Neutral red

Neutral red (NR) is a pH-sensitive dye that has been used to study proton release inside of chloroplasts, thylakoids, and chromatophores. Use of neutral red in measurement of proton release associated with oxygen evolution [10] led to the first report of the presently accepted pattern associated with the Kok’s S-scheme [11]. The technique was further refined by Junge and colleagues [12], and extended in pioneering work on the chromatophore system by Mulkidjanian [13] and colleagues. We will use neutral red to measure the kinetics of proton release in chromatophores. Through use of inhibitors we will be able to separate out different partial processes in the proton release, and correlate those with electron transfer events. This will provide insight into the roles played by specific residues the $bc_1$ complex, as it will allow us to more closely relate the effects of mutations of those residues with specific partial processes.
1.6 Figures

Figure 1.1: Structure of $b_{c1}$ monomer. Coordinates are taken from PDB ID 2QJP and visualized using VMD 1.8.1. The backbones of the three subunits are presented in cartoon representation: cytochrome $b$ (blue), cytochrome $c_1$ (red), iron-sulfur protein (green). Inhibitors are colored yellow and show the positions of the active sites: antimycin (top right) in the Q$_i$-site and stigmatellin (bottom left) in the Q$_o$-site. The 2Fe-2S cluster is purple, residue E295 is gray, and the hemes are black (from top to bottom: heme $b_H$, heme $b_L$, heme c).

The modified Q-cycle. Quinol binds at the Q$_o$-site, forming a complex with ISP. (1) The quinol transfers a hydrogen to the ISP, becoming a semiquinone. (2) The ISP rotates to the $c$ subunit, (3) reduces heme $c_1$, and releases a proton into the P-phase. Heme $c_1$ will reduce oxidized $c_2$ if any is available. The semiquinone (4) reduces heme $b_L$ and transfers a proton, likely to E295 or Y147. (5) The proton is believed to pass down a water chain to the bulk solvent while the electron passes from heme $b_L$ to heme $b_H$. (6) $b_H$ reduces quinone to a semiquinone, taking up one proton from the N-phase. The Q$_o$-site exchanges quinone for quinol, and the ISP moves back to the Q$_o$-site. A second turnover of the Q$_o$-site provides the second electron needed to reduce the Q$_i$-site semiquinone to a quinone, which exchanges for a quinol, returning the complex to its original state.
1.7 References


Chapter 2: Methods

2.1. Mutagenesis, cell growth, and chromatophore production

Chromatophores were prepared using *Rb. sphaeroides* strain BC17. This strain is a knock-out for the *fbc*-operon (which contains the genes for the three core subunits of the *bc₁* complex). We can generate a mutant copy of the *fbc*-operon and introduce it to the bacterium in a plasmid. Direct transformation of *Rb. sphaeroides* is not possible, so the plasmid is introduced via conjugation with *E. coli* strain S17. The conjugation plasmid pRK415 [1] is too large to perform mutagenic PCR efficiently (~14 kb), so mutagenesis must be implemented using the smaller (6.4 kb) pGB plasmid [2] containing a WT version of the *fbc*-operon. The mutant *fbc*-operon is excised and ligated into the larger plasmid, which is then used to transform S17. In this study, point mutations were introduced into the *b*-subunit of the *bc₁* complex for three residues: N279, R94, and Y147. The importance of these residues will be discussed in chapter 4.

Cells were grown anaerobically in Sistrom’s medium with 1 μg/mL tetracycline at 30 °C under low-light conditions. Cells were disrupted using a French press at 850 PSI. The cell lysate was centrifuged to remove large debris, and then ultracentrifuged to collect the chromatophores. The final pellet was resuspended in a pH 7 buffer containing 0.1 M KCl, 50 mM MOPS, and 30% glycerol and stored at -80 °C.

2.2. Spectroscopy

Measurements were taken using a single-beam spectrophotometer. Samples were activated by saturating flashes from a pair of xenon lamps. Three different types of measurements were made of the system.

First, we measured the movement of electrons through the photosynthetic chain. The states of various redox centers can be determined by measuring the correct wavelengths or wavelength
pairs [3]. The redox state of the bacterial reaction center was measured at 542 nm. The redox states of components of the bc1-complex were measured by subtracting wavelength pairs: cytochrome $b_{h}$ (561 nm – 569 nm); cytochrome $b_{l}$ (566 nm – 575 nm, corrected for contributions from heme $b_{h}$); cytochrome $c$ (551 nm – 542 nm).

Second, we measured the change in electrical potential across the membrane, $\Delta \psi$. Membrane-spanning carotenoids undergo an electrochromic color change in response to changes in the transmembrane potential, which we can measure at 503 nm. The details behind this process are discussed further in chapter 3.

Third, we measured protons being released into the interior of the chromatophores. Measurements were made by performing the same experiment with and without the pH-sensitive dye neutral red, and subtracting the latter from the former. Bovine serum albumin (BSA) was used as a non-penetrating pH buffer, so that the increase of pH in the exterior medium would not interfere with our measurements of the decrease of pH in the interior of the chromatophores. The difference peak for neutral red is at 531 nm, but the peak is very broad. We chose to measure at 542 nm, as that would allow us to monitor reaction center oxidation with the same measurement. To minimize variations between the +dye and –dye traces when only a single set of conditions was being tested, measurements were made without dye, then dye was added (25 μM final concentration) and the measurements were repeated. If multiple conditions were being tested (e.g. sequential addition of inhibitors), a double volume of sample was prepared and split in two, dye was added to one sample, and then the experiment was repeated for both preps.

2.3 Experimental conditions

All experiments were performed in a buffer containing 0.1 M KCl, at pH 7 unless stated otherwise. Experiments measuring only electron transfer kinetics used 50 mM MOPS as a pH
buffer. Experiments measuring neutral red color change instead used 25 mg/mL BSA. The choice of pH buffer had no discernable effect on $bc_1$ complex functioning as judged by electron transfer kinetics or electrochromic changes. 5 μM DAD, PES, and PMS were added as redox mediators; 200 μM FeEDTA was used as a redox mediator and redox buffer. Except in experiments where the electrochromic shift was measured, 5 μM valinomycin and 0.1 μM nigericin were included. Valinomycin collapses the transmembrane potential, preventing the electrochromic shift from obscuring absorbance changes due to the redox centers. In neutral red experiments, valinomycin greatly enhanced the magnitude of color change (Figure 2.1.A). In the absence of valinomycin, the proton gradient is initially from charge transfer across the membrane to generate a transmembrane potential. Because of the small capacitance of the membrane, transfer of only a few charges will generate a substantial voltage. The backpressure then inhibits electron transfer. In the presence of valinomycin, the membrane potential is collapsed by transfer of $K^+$ down the gradient, releasing the inhibition. The proton gradient then builds up as a pH gradient, requiring translocation of many more protons across the membrane, enough to swamp the buffering power. One consequence of this, however, was that due to the much larger pH gradient, the relaxation time changed from tens of seconds to at least ten minutes. Addition of 0.1 μM of nigericin, which exchanges $K^+$ for $H^+$, had no discernable effect on pH on the timescale of individual traces, but allowed the system to fully relax much more rapidly, allowing traces to be averaged with relatively short times between groups of flashes (Figure 2.1.A). Addition of 5 μM nigericin was sufficient to rapidly collapse the pH gradient (Figure 2.1.B).

Experiments were performed under poised redox conditions. The potential was adjusted using small amounts of dithionite (to reduce) or potassium ferrocyanide (to oxidize), and pH was
adjusted using small aliquots of 1M HCl and NaOH. These were added using a Hamilton syringe, and a flow of argon gas over the sample maintained anaerobic conditions.

Our experiments used significantly more BSA than the 1-2 mg/mL used in similar experiments [4-7]. We found that poising the pH and potential simultaneously was extremely difficult at these concentrations. Adding NaOH increases the pH while decreasing the potential, HCl does the reverse, dithionite decreases both potential and pH, and potassium ferrocyanide increases both. Using a higher concentration of BSA buffered the pH sufficiently that we could reliably poise both the potential and pH. As an unexpected benefit, this also increased the amplitude of neutral red color change, significantly improving our signal-to-noise ratio.
2.4 Figures

Figure 2.1: Effect of uncouplers on NR color change. A) Addition of valinomycin increased the magnitude of color change, at the cost of increasing the relaxation time needed between measurements. A tiny amount of nigericin allowed relaxation time on the scale of tens of seconds without causing relaxation on the timescale of measurements. B) Addition of a larger amount of nigericin rapidly collapsed the pH gradient.
2.5 References
Chapter 3. Characterization of proton release for WT $bc_1$ complex

Before using NR to study mutant forms of the $bc_1$ complex, we first needed a general understanding of how NR responds to turnover of the WT $bc_1$ complex in our chromatophore system, in order to design the experiments that would specifically look for perturbations in proton release via the water chain.

3.1 pH quantification

When chromatophores with neutral red present are subject to repeated flashes, the amplitude of color change varies based on flash number and starting pH. The dye’s response to a given $\Delta$pH depends on the $pK_a$ of the dye and on the current pH. Because the pH of the chromatophore interior decrease with each turnover, the dye will respond differently to each flash, even for a constant $\Delta$pH.

We can use this phenomenon to quantify the $\Delta$pH per flash. We performed the measurement at a range of pHs, and for each plotted the cumulative color change versus flash number (Figure 3.1). Starting with the second-highest pH, we offset the data from the origin until it aligned with the data from the previous pH. Offsetting by the same number of flashes each time gave the best fit for the data. The $\Delta$pH per flash appeared to be consistent within each experiment, but significantly different for the two different chromatophore preparations tested. Raising the pH by 1 unit took 24 flashes in one case and only 9 flashes in the other case (~0.04 and ~0.11 pH units/flash, respectively). The pH change per flash should vary with chromatophore interior volume and the number of active $bc_1$ complexes per chromatophore, but a factor of three difference between chromatophore preps is unexpected.
3.2 $pK_a$ shift of neutral red in chromatophores

The $pK_a$ of neutral red is 6.8 in solution, but it is known to shift when the dye enters the membrane. The interaction between the charged form of the dye and the charged head groups of lipids in the membrane stabilizes the protonated form, shifting the $pK_a$ to be more basic [1]. The data from the preceding experiment can be used to characterize this $pK_a$ shift. We calculated the $DI/I$ per flash, and converted the x-axis from flash # to pH with the assumption of constant $\Delta pH$ per flash. The data was plotted and then fit with a Gaussian (Figure 3.2). Despite the significant difference in $\Delta pH$/flash for the two chromatophore preps, the $pK_a$ was 7.1 according to both datasets.

3.3 Effect of $bc_1$ complex inhibitors on neutral red color change

There are two paths for proton release from the $Q_o$-site, each correlated with an electron transfer. The first proton and electron transfer together to reduce the ISP$_{ox}$ to ISPH, leaving SQ$_o$ as intermediate product, and the proton is released when the ISPH reduces cyt $c_1$. The second electron transfers from SQ$_o$ to heme $b_L$ and then to heme $b_H$, while the proton is thought to exit the $Q_o$-site via a water chain which is stabilized in part by one of heme $b_L$’s propionates. We can, in principle, predict the number of protons released via each path using what is currently known about turnover of the $bc_1$ complex.

More formally, the partial processes involved can be defined by reaction equations.

Flash excitation ($\cdot \cdot \cdot \cdot$) generates two oxidizing equivalents for each $bc_1$ complex monomer, initially oxidizing cyt $c_2$.

$$2P + 2 \text{cyt } c_2 \cdot \cdot \cdot \cdot \Rightarrow 2P^+ + 2 \text{cyt } c_2 \Rightarrow 2P + 2\text{ferricyt } c_2$$
In the presence of myxothiazol (myxo) (or of asoxystrobin), one proton is released on oxidation of ISPH (pK_{red} \sim 12.5 in the initially reduced high potential chain), by ferriheme c_1 generated after the flash:

$$E.(\text{myxo}).\text{ISPH.ferroc}_1 + 2\text{ferricyt} \leftrightarrow E.(\text{myxo}).\text{ISP}_{\text{ox}}.\text{ferri}_1 + 2\text{ferrocytc}_2 + H^+$$

With stigmatellin (not shown), no H^+ release is seen, consistent with binding of ISPH in a tight complex with the inhibitor.

In the absence of Q_o-site inhibitors, with the quinone pool half-reduced, most bc_1 complexes have the g_x = 1.80 complex (Q.ISPH, an enzyme product (EP-) complex) at the Q_o-site. This is replaced by the ES-complex when ISP_{ox} is generated as oxidizing equivalents reach the complex [2]. In the presence of antimycin, with the two b-hemes initially oxidized, 2 H^+ are released on oxidation QH_2. The first is that from oxidation of ISPH (as above), but ISPH is regenerated in the first electron transfer on oxidation of QH_2 by ISP_{ox}:

$$g_x=1.80 \text{ (EP-)complex} \rightarrow \text{ES-complex}$$

$$E.Q.\text{ISP}.\text{ferroc}_1 + 2\text{ferricyt} \leftrightarrow E.Q.H_2.\text{ISP}_{\text{ox}}.\text{ferri}_1 + Q + 2\text{ferrocytc}_2 + H^+$$

**intermediate complex**

$$E.Q.H_2.\text{ISP}_{\text{ox}}.\text{ferri}_1...b_L.b_H \leftrightarrow E.Q.H^+.\text{ISP}.\text{ferri}_1...b_L.b_H$$

The second H^+ comes from oxidation of QH^+, the neutral SQ intermediate generated in the first electron transfer, by heme b_L, which is then rapidly oxidized by heme b_H.

$$E.Q.H^+.\text{ISP}.c_1...b_L.b_H \leftrightarrow E.Q.\text{ISP}.c_1...b_L.b_H$$

$$E.Q.\text{ISP}.c_1...b_L.b_H \leftrightarrow E.Q.\text{ISP}.c_1...b_L.b_H + H^+$$
Further turnover is then arrested as a result of the much lower value of $K_{eq}$ for reduction of heme $b_L$ ($K_{eq} \sim 3$) than for heme $b_H$ ($K_{eq} \sim 400$). The oxidizing equivalent in the high potential chain is shared between ISP and heme $c_1$: $\text{ISP}_{\text{ox}}.\text{ferro}c_1 \rightleftharpoons \text{ISPH.ferri}c_1$, $K_{eq} \sim 3$.

In the absence of any inhibitor, ferroheme $b_H$ generated as the first QH$_2$ turns over, is oxidized at the Q$_i$-site. After exchange of product Q, by QH$_2$ at the Q$_o$-site, a second QH$_2$ is oxidized, consuming the second oxidizing equivalent generated in the photochemistry, and releasing 2 H$^+$ to the P-phase.

$$
\text{QH}_2 + E.Q.\text{ISP}.c_1...b_L. b_H.Q \rightleftharpoons E.QH_2.\text{ISP}_{\text{ox}}.c_1...b_L. b_H.Q^+(H^+) + Q
$$

$$
E.QH_2.\text{ISP}_{\text{ox}}.c_1...b_L. b_H.Q^+(H^+) \rightleftharpoons E.Q.\text{ISP}.c_1...b_L. b_H + \text{QH}_2 + 2\text{H}_P^+
$$

Except for the H$_P^+$ generated on oxidation of ISPH, which is released with $t_{1/2} \sim 150$ μs, all rates of H$^+$ release measured in wildtype reflect the rate-limiting first electron transfer, convoluted with turnover. Since electrogenic processes and release of the other protons occur after the first electron transfer has generated SQ$_o$, the intrinsic rates of subsequent partial processes must all be fast compared to the limiting rate observed. The second turnover follows the first without any hiatus, showing that exit of product Q, and replacement by QH$_2$ must also be rapid compared to the limiting step.

We can determine the number of protons released in the presence of inhibitors by comparing the data to that of the uninhibited complex. The pH dependence overall can be understood in terms of the apparent pK of neutral red, and the assumption that release of protons from complete oxidation of QH$_2$ follows the expectation of the reaction equation. Because full turnover at any pH returns the complex to the initial state in the dark interval between flashes (~30 s), the uninhibited complex will release the 4 protons into the interior of the chromatophore expected from the reaction equation, and we can simply normalize the inhibited traces to this value.
Even if we had a perfect model, we would not expect total agreement between prediction and data. Two important variables are the ratio of RC to $bc_1$ complex monomers, and variability introduced by heterogeneity in the chromatophores. However, we can still use these predictions as a baseline, and see if there are larger-scale discrepancies with the data that cannot be explained by a heterogeneous sample.

3.3.1 $Q_0$-site inhibitors

The simplest case to interpret is in the presence of stigmatellin. Stigmatellin binds in the distal region of the $Q_0$-site, in a complex with ISP so tightly bound that no release occurs on oxidation of the HPC. The $Q_0$-site is unable to turn over, and the ISP will not release any protons. We predict that there would be no proton release, and our data show negligible color change (equivalent to $\sim 0.1$ protons) at all pHs (Figure 3.3.A).

A second class of $Q_0$-site inhibitors, typified by myxothiazol and MOA-type inhibitors like azoxystrobin, bind in the proximal volume of the site, and displace all quinone species which bind more weakly. This leaves the ISP free to interact with cyt $c_1$. Under conditions tested, the ISP will be fully reduced (ISPH), and thus protonated ($pK_a > 12$). The two oxidizing equivalents generated by the flash will fully oxidize the HPC, causing a fraction of complexes to release a proton. The $pK_a$ of oxidized ISP in the isolated protein is 7.6, and we might expect this value to apply in the presence of azoxystrobin, so we would expect to see significant variation over the pH range tested (pH 7-8).

On flash excitation in the presence of azoxystrobin at pH 7 and 7.25 the trace has the expected shape, although with larger magnitude than predicted (Figure 3.3.B). At pH 7.5 and above there is still net proton release (Table 3.1). However, we also see an immediate negative color change, indicating basification of the chromatophore interior (Figure 3.3.C). Taking account
of the uptake, the trend is as expected, but with a displacement as a function of pH from the predicted value. A simple interpretation would be that the ISP under these conditions has a $pK_{\text{ox1}}$ \textit{in situ} different from that in the isolated protein by $\sim -0.4$ units. The only comparative data we know of are from Ugulava et al. [3], but the slope of the pH dependence in this range is not well enough defined to test for a difference.

An important question for analysis of the uninhibited and antimycin-inhibited data is whether the uptake of internal protons (basification) occurs only in the presence of azoxystrobin or if it is a general feature of the system. The kinetics of the color change indicate that it is the former. The basification reaches its maximum extent within 20 $\mu$s and immediately begins recovering, with a half time of $\sim 100$ $\mu$s. In both the uninhibited and antimycin-inhibited complex, there is no color change until 100+ $\mu$s, at which point acidification begins. Although we are unsure what is causing this phenomenon, which has an apparent $pK \sim 7.7$, we are confident that it does not complicate analysis of other inhibition states.

The kinetics of the proton uptake (immediate uptake ($< 10 \mu$s) followed by rapid relaxation) suggest that the phenomenon is connected to $\Delta \psi$ across the membrane, generated in the photochemistry during the 5 $\mu$s flash. Although the valinomycin present rapidly dissipates the potential difference, there is still a short period immediately after the flash where the $\Delta \psi$ generated is significant. The direction of the electric field would serve to push protons into the membrane, or into the aqueous-accessible portions of the $bc_1$ complex. A residue with an appropriate $pK$ could become protonated due to the local enrichment of protons. Given that proton uptake does not occur in the presence of stigmatellin, it follows that this hypothetical residue is unavailable in that state. Structures show E295 H-bonded to the inhibitor, but free in the presence of azoxystrobin, so E295 would provide a plausible candidate, and the water chain a channel.
3.3.2 Qo-site inhibitor antimycin

Proton release in the presence of the Qo-site inhibitor antimycin in more complicated to predict. Although two oxidizing equivalents are available to the Qo-site after the first flash, the complex does not turn over twice. Because of the low equilibrium constant for electron transfer from the semiquinone to heme bL, the Qo-site reaction will not go to completion unless heme bH is available to oxidize heme bL. Heme bH remains reduced after the first turnover in the presence of antimycin, and the fraction expected to turn over a second time reflects low value of $K_{eq}$ for reduction of heme bL when the HPC is already half-reduced. This fraction depends on the stoichiometry of RC with respect to $bc_1$ complex monomer. In the “ideal” case of Rc:$bc_1 = 2$, the fraction is ~0.15, but stochastic population of the chromatophores by redox complexes, the experimental result will reflect a statistical range of values for Rc:$bc_1$ about the mean measured. In the “ideal” case, 1.15 protons would be released to the HPC. Comparison with the azoxystrobin results is complicated by a new consideration. With azoxystrobin present, the site cannot form the ES-complex because QH2, the substrate cannot bind. In the absence of azoxystrobin, the ES-complex can form with ISPox is the second substrate, but only participates in the dissociated form. Consequently, any ISPoxH+ which had a proton bound initially, will release it. The complications in considering $pK_{ox1}$ in the azoxystrobin case no longer apply. We might therefore expect that, for full oxidation of QH2, a total of 2.3 protons could be released to the P-phase. However, in considering pH dependence, we have to consider an additional complication; the midpoint potential for cyt $c_1$ is 270 mV, independent of pH, and that of the ISP decreases from 300 to 280 over the pH range 7-8. Consequently, the electron will be shared between them in a pH-dependent manner. Reduced ISP will always be protonated ($pK_a > 12$), while oxidized ISP will be partially protonated. Exchange of product Q for QH2 will allow formation of the ES-complex ($pK_a = 6.6$ in
the absence of Q$_o$-site inhibitors). Thus, as pH increases, there will be less ISPH, and smaller proportion of oxidized ISP$_{ox}$, both resulting in greater proton release.

On completion of the Q$_o$-site turnover, oxidation of SQ$_o$ will also release 1.15 protons to the water chain in equilibrium with the LPC. Our preliminary hypothesis was that interaction of the H$^+$ with reduced heme b$_L$, either through coulombic attraction, or through a change in pK$_a$ of the heme propionate, might prevent the proton from exiting the water chain. The proton from the first turnover would be released to the bulk solvent (because heme b$_L$ is reoxidized), while the proton from the second transfer would be retained.

Following the second flash, introduction of two additional oxidizing equivalents to the HPC will drive the reaction forward and allow further (though still not complete) turnover on reduction of heme b$_L$. The release of protons via reactions in the HPC will depend on considerations discussed above with respect to the first turnover; the protons retained by oxidized ISP will decrease with pH, as after the first flash. However, the oxidizing equivalents introduced into the partly oxidized HPC will release the protons retained by reduced ISP after the first flash, and any protons from ISPH generated from the additional turnover of the Q$_o$-site. This proton released on oxidation of SQ$_o$ will be delivered to the water chain, but we might expect these would stay bound to the propionate of the reduced heme b$_L$. The HPC will be fully oxidized. The protons release expected after the second flash might show a small pH dependence, with an increase over the range above pH 7.0, compensated by a decrease above pH 7.5 (Figure 3.4.D, green trace).

The data following the first flash excitation show, in the presence of antimycin, the release of close to the 2 H$^+$ expected from naïve assumptions on the oxidation of a single QH$_2$. The proton release in the presence of antimycin has a pH-dependence after both the first and second flashes (Figure 3.4). Although we predicted an increase in proton release with starting pH after the first
flash, the calculated value consistently overestimates the number of protons release, and underestimates the magnitude of the pH-dependence. For the second flash, while the proton release predicted lies in the middle of the values observed, we see an unexpected pH-dependence similar to that after the first flash.

An explanation for the underestimation for the first flash is that we were mistaken to assume that the proton passed to the water chain during the first turnover was released from the complex, and the proton is in fact retained in a pH-dependent manner.

**3.4 Isolation of partial processes**

A single flash in the absence of inhibitors results in two turnovers of the $bc_1$ complex, which follow a continuous trajectory, and are therefore impossible to separate through direct observation of one reaction component. In the photochemically driven process here, oxidation and reduction kinetics of the redox reactants of RC and $bc_1$ complex ($QH_2$ and cyt $c_2$) are convoluted. We can measure the full turnover from protons release or transfer of charge across the membrane. Furthermore, through use of inhibitors we can isolate the kinetics of the first and second turnovers for both proton release and electron transfer.

The first turnover of the $Q_o$-site can be measured directly by observing the reduction of heme $b_H$ in the presence of antimycin (Figure 3.5.A, red trace). To determine the second turnover we can take advantage of the electrochromic absorbance change at 503 nm in carotenoids in response to a change in the transmembrane voltage [38]. In the full turnover of the photosynthetic chain in chromatophores, electrons crossing the membrane through the $bc_1$ complex generate a significant portion (ideally 50%) of the voltage difference developed in response to a flash. Subtracting the absorbance change in the presence of inhibitors from that without inhibitors, we can determine the kinetics of two electrons crossing the membrane via the LPC (Figure 3.5.B,
black trace). By normalizing $b_H$ reduction to a value of 1 and the electrochromic shift to two, we can subtract the former from the latter and isolate the kinetics of the second turnover of the Q$_{0}$-site (figure 3.5.C, blue trace).

The method for separating the proton release of the first and second turnovers is similar. We expect the proton release in the presence of antimycin to be predominantly from a single turnover of the Q$_{0}$-site. Comparing the uninhibited and antimycin-inhibited traces (Figure 3.5.B, black and red traces, respectively), we see a very close match between both the rate and timing of the initial neutral red color change, suggesting that the antimycin-insensitive proton release is the same as the proton release from the first turnover in the absence of inhibitors. By subtracting the antimycin trace (proton release from first turnover) from the uninhibited trace (proton release from two complete turnovers) we isolate the kinetics of proton release from the second turnover (Figure 3.5.B, blue trace).

To simplify discussing these partial processes, we will refer to them as E1, E2, P1, and P2, which denote the electron transfer or proton release associated with the first or second turnover (Figure 3.5.C and 3.5.D). Note that while E1 and E2 each represent a single event (an electron passing to the LPC), P1 and P2 are more complicated. First, each turnover releases two protons which can be measured (the first passed to ISP and the second to the water chain). Thus P1 and P2 refer to all of the protons released by the first and second turnovers, respectively. Second, protons released in the presence of antimycin include what is released prior to Q$_{0}$-site turnover (see previous section about azoxystrobin), and a small number of complexes do undergo a second turnover. On the other hand, the proton passed to the HPC during the first turnover is partially retained by the ISP, and it is unclear if the proton passed to the water chain is able to easily exit the complex while heme $b_H$ is reduced (see previous section about antimycin). Regardless, as we
will discuss next, we find an excellent match between the kinetics of E1 and P1, and an interesting relationship between P2 and E2.

For both proton release and electron transfer, there is a pH-independent initial delay of ~150 μs after the flash. This is approximately the time expected for the diffusion of oxidized $c_2$ from the RC to the $bc_1$ complex. Neutral red appears to respond rapidly to proton release, as there is no discernable delay between initial proton release and initial electron transfer observed. The delay between the first and second turnovers is difficult to measure precisely as there is a “ramp-up” period before the process reaches its maximum rate. However, there is still good agreement between P2 and E2. This delay is pH-sensitive, ranging from 0.4 ms at pH 8 to 1 ms at pH 7. The rate of $Q_0$-site turnover is known to be pH-dependent [4]. As the rate of turnover increases, it takes less time for the $Q_0$-site to return to its initial state so it can begin the second turnover.

While the initial rate of E1 and P1 are in good agreement, proton release is significantly slower than electron transfer for the second turnover at pH 7. As pH increases, the rates for all 4 processes increase, with the most significant increase for P2. Significantly slower than E2 at pH 7, by pH 8 it has the same rate. It is possible that this is related to the pH dependence for protons released in the presence of antimycin (Figure 3.4).

3.4.1 Separation of turnover-independent proton release from “1st” turnover

As stated previously, what we have been referring to as P1 includes the protons released by turnover of the $Q_0$-site as well as the protons released prior to turnover. It would be useful if we could separate the two processes. Initially, we assumed we could subtract the azoxystrobin trace from the antimycin trace, and the turnover-independent proton release in the presence of azoxystrobin would cancel out the pre-turnover proton release in the presence of antimycin. The
first complication arose when we observed the pH-dependent basification of the chromatophore in the presence of azoxystrobin. We concluded that this phenomenon only took place in the presence of azoxystrobin (see section 3.2.2), and thus would introduce an error if we subtracted it from the other traces.

We later realized that we would not expect the subtraction to work even at a pH where basification did not take place. The behavior of ISP_{ox} will be different based on the presence or absence of azoxystrobin. If it is present, then the ISP will be unable to bind to the Q_{o}-site, and thus is expected to have the pK_{a} = 7.6 of the isolated protein. Conversely, in the absence of azoxystrobin, the ISP will be able to bind to the quinol and form the ES complex. The number of protons released on oxidation of the HPC will be different in the two situations, so subtraction will not isolate the kinetics of proton release from the first quinol oxidation.

However, if you plot the 3 inhibition states (No inhibitor, antimycin, antimycin + azoxystrobin) on the same axes, there is good agreement in initial rate (Figure 3.6). Subtracting to isolate the partial processes also yield reasonable results. We noted previously that the color change in the presence of azoxystrobin is greater than expected, based on the assumption of a pK_{a} for the ISP_{ox} of 7.6. If the actual value was close to the value of 6.6 for the uninhibited state, then the predicted proton release would be more in line with the value measured, and the point raised in the preceding paragraph about the mismatch between Q_{o}-site-independent proton release in the presence or absence of azoxystrobin would no longer apply.
3.5 Figures

**Figure 3.1: Color change after repeated flashes.** Chromatophores were subject to repeated flashes and the cumulative color change ($\Delta I/I$) after each flash was plotted against flash #. The data for pH 7.75 was offset from the origin to align with the pH 8.00 data. This was repeated for pH 7.50-7.00, resulting in a characteristic titration curve. The number of flashes needed to raise the pH by 1 unit was different for the two chromatophore preps tested: 24 flashes (left) and 9 flashes (right).
Figure 3.2: pK$_a$ shift of neutral red in chromatophores. The color change per flash (ΔI/I) was calculated from the data in figure 1, and flash # was converted to pH. A Gaussian was fit to each data set. Although the two chromatophore preps tested had very different ΔpH/flash, both showed a pK$_a$ of 7.1 for the dye responding to acidification of the chromatophore interior.
Figure 3.3: Color change in the presence of Qo-site inhibitors. A) There is negligible proton release in the presence of stigmatellin. B) There is noticeable, though significantly reduced proton release in the presence of azoxystrobin. C) Net proton release is always observed in the presence of azoxystrobin, but at higher pHs there is also an initial uptake of protons.
Table 3.1: Proton uptake and release in the presence of azoxystrobin. Net proton release was observed at every pH, but at higher pHs there was also an initial uptake of protons. Combining the two we can calculate the total number of protons released, although it is unclear how much of this is the expected release of protons from ISPH, and how much is re-release of the protons taken up initially. The predicted proton release was calculated assuming a $pK_a$ of 7.6 for the oxidized ISP in the presence of azoxystrobin.
Figure 3.4: Proton release in the presence of antimycin. (Top) A pH-dependence was observed for release of protons after both the first and second flashes. (Bottom) The pH-dependence observed was greater than predicted (green trace; other colors indicate different chromatophore preps, and each line is a separate experiment).
Figure 3.5: Isolation of partial processes. (Top) Antimycin limits the Q$_o$-site to a single turnover, so subtracting that trace from the uninhibited trace isolates the electron transfer or proton release from the second turnover. (Bottom) Plotting the proton and electron transfers for a single pH together, we see a good agreement for the first partial processes, and a pH-dependent agreement for the second partial processes.
Figure 3.6: Subtraction of myxothiazol. (Left) Matching initial rates suggests that the same initial process is taking place in each inhibition state. (Right) Subtracted traces appear to show that there the three processes (initial proton release, 1st turnover, 2nd turnover) are separable by this technique.
3.6 References
Chapter 4: Investigation of water chain mutants

4.1 Potentially important residues identified by simulation

Residues N279 was initially chosen on the basis of the first MD simulation of the $bc_1$ complex [1], which showed the water chain leading from the $Q_o$-site to the P-phase, and recognition that this might important in proton processing [2]. Examination of the structures and alignment of sequences showed that N279 was likely to be in H-bonding contact with the chain. These features were later confirmed when crystallographic waters were identified in higher resolution structures [3], and when *Rhodobacter* structures became available [4,5].

Crystal structures showed that R94 H-bonds the heme $b_L$ propionate closest to $Q_o$-site. MD simulation revealed a dynamic picture in which R94 can H-bond with either propionate, and much of the time with both. Rotation about the propionate carboxylate bond switches the O-atom involved back and forth. The volume between N279, R49, Y147, and E295 hosts a dynamic water population with H-bonding extending to the near propionate, and through the protein to the P-phase (Figure 4.1).

Barragan et al. produced a simulation examining substrate binding at the $Q_o$-site [6]. Crystal structures with stigmatellin bound at the $Q_o$-site show that E295 is hydrogen bonded to the inhibitor, supporting a model in which E295 is bound to the quinone and acts as the primary acceptor for the second proton transfer. In this simulation, where stigmatellin was replaced with a quinone, the hydroxyl group of Y147 acted as an intermediary between the E295 and the QH$_2$, forming stable hydrogen bonds with both. Furthermore, during the quantum chemical optimization procedure, the hydroxyl group of Y147 donated its hydrogen to E295, becoming available to accept proton transfer from the quinol.
4.2 Previous studies on chosen residues

Working with the isolated protein in detergent solubilized state of liposome bond, Qu et al. examined the effect of mutations at this site [7]. Steady state turnover in R94N was 51% of WT, 35% for R94D, and 4% for R94A. Half-time for reduction of heme \( b_H \) in the presence of antimycin increased by factors of 1.81, 2.56, and 4.76. (Inverse half-time give 55%, 39%, and 21% rates compared to WT.) The proton-pumping ratio was essentially unchanged for R94N (1.48 compared to 1.5 for WT), but noticeably reduced for R94A and D, (1.2 and 1.23, respectively.)

Saribaş et al. generated several Y147 mutants in \( R. \) capsulatus, and tested them using chromatophores [8]. Substitutions caused moderate to severe inhibition, with the rate of \( b_H \) reduction at 40% of WT for Y147 F&V, and only ~5% for Y147 A&S. Y147V was also found to be myxothiazol resistant (only the V and F mutants were tested for myxothiazol sensitivity).

4.3 Experimental Results

The normalization of NR traces was modified slightly from the work presented in the previous chapter. We were concerned that variations of RC/bc1 stoichiometry between chromatophore preps would introduce errors when comparing mutants. To make comparisons more straightforward, we normalized the NR trace in the presence of antimycin to 1, and used that normalization constant for the other NR traces for that experiment.

We found that all mutants are inhibited for all partial processes (Figure 4.2). However, we are much less interested in the degree of inhibition in individual processes than we are in the pattern of inhibition compared to WT. For WT, the kinetics of P1 and E1 match closely, and P2 has about 1/3 the rate of P1. N279Y is inhibited compared to WT, and R94Q is even more inhibited, but the relationship between P1, P2, and E1 remain the same (Figure 4.3). The other mutants show more interesting behavior (Figure 4.4).
The effect of R94A, Y147T, and N279F on the relation between P1 and P2 closely matches one of our earliest hypotheses for water chain mutants. We predicted that the second proton needed to rapidly exit the complex in order for subsequent turnover of the Q_{o}-site to proceed normally. If the proton was unable to escape promptly, then it could interfere with the following turnover, and this would be visible as an increased inhibition for the second turnover compared to the first. In WT and the other mutants, P2 is typically ~1/3 as fast a P1, but for these two mutants the value is ~1/6. This difference is particularly striking for the R94 mutants, which have virtually identical kinetics for E1 and P1, but for P2 R94A is substantially slower.

The relation between E1 and P1 for N279F and Y147T is more difficult to explain in terms of those mutations interfering with the second proton exit specifically. For WT and the other mutants, there is a close match between the kinetics of E1 and P1, but for these two mutants E1 is significantly inhibited compared to P1. While the mismatch in initial rates could indicate that the second proton and electron transfers are inhibited compared to the hydrogen transfer to the ISP, we would expect a sharp decrease in the slope of P1 after an initial fast rise. Instead P1 is rather smooth, and stays ahead of E1. This would suggest that the second proton is able to pass down the water chain and reach the bulk solvent before the second electron transfer occurs.

The R94 mutants show an interesting behavior in the presence of myxothiazol (Figure 4.5). While the neutral red kinetics for N279F appear almost identical to WT, R94Q shows no color change, and R94A shows a slight basification in response to flash activation. (Y147T is myxothiazol resistant.) As was the case with the pH-dependent basification observed in the WT in the presence of azoxystrobin, it is unclear why this occurs. While it might seem obvious to suggest that they are the same phenomenon and the R94 mutation simply shifts the pK of the effect, the difference in kinetics suggests against that connection. The shape of the curve in WT was roughly
similar at all pHs, simply displaced downward. Also, at all pHs there was still net proton release. The R94 mutants, by contrast, have very flat kinetics.

4.4 Summary

These results support the idea that residues N279, R94, and Y147 play a role in release of the second proton from the Qo-site turnover. For each residue there was a mutant (N279F, R94A, Y147T) that was more inhibited for the second turnover than the first, as predicted for mutants where the escape of the second proton from the site is impaired. N279F and Y147T also show a significant deviation between electron transfer to heme $b_H$ and proton release for the first turnover.
Figure 4.1: Water chain residues. Residues mutated in this study are shown, along with E295 and heme b$_L$. The four residues are able to interact with the heme propionate closest to the Qo-site (through direct H-bonding for R94, or through a single intermediary water for the others), but in this image are shown playing other roles. E295 and Y147 are oriented towards the Qo-site to the left where they likely play the role of primary proton acceptor during quinol oxidation. R94 is H-bonded to the heme propionate further from the Qo-site. N279 is interacting with waters at the opening of the channel that leads to the bulk solvent. The grey surface indicates the volume filled by water, with representative water molecules shown inside. Structure taken from an MD simulation based on PDB ID 2QJP.
Figure 4.2: Partial processes. Comparison of WT and mutant reveals significant differences in inhibition of specific partial processes.
Figure 4.2: WT and “WT-like” mutants. In WT, the kinetics of proton release and electron transfer for the first turnover match closely. Proton release from the second turnover is slower by a factor of three. N279Y and R94Q are inhibited, but all partial processes are inhibited to the same degree, so the relationship between partial processes remains the same.
Figure 4.3: “WT-unlike” mutants. These mutations deviate from the WT in terms of the relationship between partial processes. For all three mutants, P2 is twice as inhibited as P1 (1/6 compared to 1/3 in WT). For Y147 T and N279F, the close match between kinetics of P1 and E1 is absent.
Figure 4.4: Effect of myxothiazol on proton release. While the kinetics for N279F are almost identical to WT, R94 mutants show no proton release or a small uptake of protons. Y147T is resistant to myxothiazol.
4.6 References


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