STRUCTURE AND FUNCTION STUDIES OF POLAR MUTANTS OF THE $Q_A$ POCKET IN THE BACTERIAL PHOTOSYNTHETIC REACTION CENTER OF *RHODOBACTER SPHAEROIDES*

BY

AIDAS J. MATTIS

DISSERTATION

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Doctoral Committee:

Professor Emeritus Anthony R. Crofts, Chair
Professor Robert B. Gennis
Professor David M. Kranz
Professor Satish K. Nair
Abstract

The bacterial photosynthetic reaction center (RC) is the protein that converts light to chemical energy. The light is initially absorbed by a pair of bacteriochlorophylls that then transfer an electron through a series of cofactors until it reaches the final two electron acceptors, the primary quinone (Q\textsubscript{A}) that then reduce the secondary quinone (Q\textsubscript{B}). In \textit{Rb. sphaeroides} these quinones are chemically identical ubiniquinones and the protein must tune the midpoint potential (E\textsubscript{m}) of each quinone to make electron transfer from Q\textsubscript{A} to Q\textsubscript{B} favorable. Using site directed mutagenesis together with the techniques of X-ray crystallography, flash kinetic spectroscopy, and quinone substitution, I was able to probe how structural changes contribute to E\textsubscript{m} changes and work to better understand the physical chemistry involved.

The mutation of the wild type (WT) Ile at the 265\textsuperscript{th} amino acid of the M subunit (M265), which lies within van der Waals contact to the primary quinone (Q\textsubscript{A}), to the polar hydroxyl (O-H) mutants of Ser (M265IS) and Thr (M265IT), previously showed a drop in the \textit{in situ} E\textsubscript{m} of Q\textsubscript{A} by 85 and 100 mV, respectively (Takahashi et al 2001). In repeating Takahashi et al’s kinetic work, it was discovered that there are two separate components for the Q\textsubscript{A} back reaction not previously recognized. The structures of the two mutants were solved using X-ray crystallography and the orientation of the M265 side chain O-H, relative to the quinone, for the two mutants are in different orientations. The M265IS O-H is located in a position where four potential hydrogen bonds (H-bonds) are present, while the M265IT O-H is positioned where the O-H has only one potential H-bond. Q\textsubscript{A} in M265IS has an additional H-bond, not present in WT, between the 2-methoxy of Q\textsubscript{A} and the backbone nitrogen of M249 that maybe necessary to stabilize the quinone due to the increase in the size of the quinone binding pocket. For both hydroxyl mutants the H-bond to the C1 carbonyl of Q\textsubscript{A} was significantly shorter than Xray-avg
(the average of all atomic distances from currently deposited RC X-ray structures with resolution better than 2.80 Å) while only the H-bond to the C4 carbonyl of QA from M265IT was significantly shorter.

The Ile at M265 was also mutated to the polar amide mutants Asn (M265IN) and Gln (M265IQ). M265IN presented kinetics not very different to M265IT, indicating that the \textit{in situ} $E_m$ of QA was similar. However, M265IQ showed a slower QA$^-$ back reaction, which is opposite from the other three polar mutants. Both mutants showed two component kinetics for the QA$^-$ back reaction that varied with pH. The QA$^-$ back reaction was also slower for M265IQ compared to WT, which is the same direction as the other mutants. These results indicate that the \textit{in situ} $E_m$ of M265IQ is likely unchanged from WT. It was further found that QA of M265IQ was only occupied approximately 50% of the time. The structures of M265IN and M265IQ were solved using X-ray crystallography. M265IN showed that the side chain only took on one conformation, but the rotamer of the side chain amide could possibly take on two orientations. M265IQ showed two conformations for the side chain of M265 consistent with one conformation of QA bound (Conf. A) and the other with QA dissociated (Conf. B) or bound at a more distant site from the WT binding position. The amine and carbonyl of the side chain of Asn-M265 showed both H-bond and repulsion with either the C4 carbonyl of QA or the δ nitrogen of His-M219 depending on the rotamer of the amide. The side chain amine of Conf. A of M265IQ has an internal H-bond with the backbone carbonyl and the side chain carbonyl has a potential H-bond to the δ nitrogen of His-M219, which bifurcates the δ nitrogen H-bond between the side chain of M265 and the C4 carbonyl of the quinone. Both mutants showed longer H-bonds between the C4 quinone carbonyl and δ nitrogen of His-M219 when compared to Xray-avg, but only M265IQ mutant showed a shorter hydrogen bond between the C1 quinone
carbonyl and the M260 backbone N. The M265IN C1 quinone carbonyl H-bond was not significantly different from Xray-avg.

The RC is a finely tuned system that tightly controls the midpoint potentials of $Q_A$ and $Q_B$ so that an electron can be favorably transferred from one ubiquinone to another. The addition of a polar group to the non-polar $Q_A$ site decreases the midpoint potential by approximately 100 mV for M265IS, M265IT, and M265IN. However, M265IQ is such a structurally large amino acid addition to the RC that the quinone is displaced 50% of the time from its WT location and gives a much more complicated kinetic picture. Based on the crystal structures of M265IS and M265IT, the orientation of the hydroxyl controls the $E_m$, but to a much smaller extent than simply the addition of the polar group to the local vicinity of the $Q_A$ site. The addition of an amine group to $Q_A$ has a similar $E_m$ change to the addition of a hydroxyl. I therefore conclude that local electrostatics are likely the largest factor in controlling the $E_m$ of $Q_A$. Electrostatic calculations are needed to calculate how adding a polar group at M265 changes the $E_m$ of $Q_A$. 
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This work is dedicated to my parents:

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Chapter 1:

General Introduction to the Bacterial Photosynthetic Reaction Center

Abstract:
This chapter introduces the general concepts of the bacterial photosynthetic reaction center, including basic information about its structure, function, kinetics and some previous mutational studies exploring the primary quinone ($Q_A$) binding site. Further, this chapter discusses previous work that used mutagenesis, kinetic flash spectroscopy, and FTIR studies to probe what controls the midpoint potential of $Q_A$.

Photosynthesis and the Reaction Center
The Sun is the main input of energy that allows life to exist on Earth. Light from the Sun is converted to chemical energy by photosynthesis. There is a large amount of evidence that photosynthesis evolved early on the timeline of life on Earth, but there is debate as to when exactly this occurred [1]. At least 3 billion years ago simple bacteria already had the machinery to perform basic photosynthesis and this process has since evolved to the various photosynthetic systems seen today in bacteria and plants.

As there is significant evidence that eukaryotic photosynthesis originated from endosymbiosis of a cyanobacterial-like organism, it is reasonable to assume that photosynthesis is homologous between bacteria and plants [2, 3]. This homology allows researchers to extrapolate the study of simpler bacterial system to explain the more complex eukaryotic photosynthetic systems. At the core of both of these systems are photosynthetic reaction centers (RC), the proteins that convert light to chemical energy.
Two classes of RCs exist, type I and type II, differentiated by their terminal electron acceptor cofactors. Type I RCs have iron sulfur clusters while type II RCs have quinone complexes [4, 5]. Oxygenic phototrophs, such as cyanobacteria and plants, e.g., spinach, have both types of RCs while anoxygenic phototrophs only possess one type, either I or II. Purple bacteria, e.g., *Rhodobacter sphaeroides* (*Rb. sphaeroides*), utilize the type II RC, while green sulfur bacteria, e.g. *Chlorobium limicola*, use the type I RC. The type II RC in bacteria has been extensively studied and has been used as a homologous model system for Photosystem II in oxygenic phototrophs, e.g. plants. This work will focus on type II RCs and any further discussion of RCs will be in reference to type II RCs unless specified otherwise.

After RCs have converted light to chemical energy intermediates, the organisms must still convert these intermediates to more stable energy sources, i.e., ATP. This conversion is performed by either the $bc_1$ or $b_6f$ complex in anoxygenic and oxygenic organisms, respectively. While oxidizing electron-carrying intermediates, both complexes ($bc_1$ and $b_6f$) pump protons across the membrane to form a proton motive force that ATP synthase can utilize for production of ATP [6, 7]. This ATP can then be immediately used as an energy source or the ATP can be used to anabolize energy storage molecules such as glucose for later use.

The first step in photosynthesis is the absorption of a photon of light by a photosynthetic pigment. Chlorophyll (Chl) and bacteriochlorophyll (BChl) are the main pigment molecules used by phototrophic organisms to harvest light energy and begin the conversion of light to stable chemical energy. Eight different Chls (a, b, c, d, f, 8-hydroxy-Chl a, divinyl-Chl a and b) and six BChls (a, b, c, d, e, and g) are known to occur in the wild [8, 9]. Chlorophyll was first reported in 1817 by French chemists while chlorophyll f was discovered only recently in 2010.
Even though other pigments can be used for light harvesting, e.g., carotenoids, all phototrophic organisms require chlorophyll for photochemical conversion [4].

Bacteria primarily use bacteriochlorophylls to capture light. These are mostly located in the light harvesting antenna complexes, LH1 (core) and LH2 (peripheral) in purple bacteria. LH1 proteins form an open ring complex around an individual RC that associates with a PufX protein. The open ring LH1 complex with RC-PufX is found in a dimer structure with a second RC-LH1-PufX complex. Two structures exist for LH2 proteins, historically dependent on illumination conditions, though both can be found under either low or high intensity light. LH2 molecules form light-capture domains that interconnect linear rows of dimers of RC-LH1-PufX complexes (high intensity light) or the LH2s can form densely packed LH2 only domains (low intensity light) [10, 11]. The light harvesting antenna complexes collect energy from photons of light and transfer the energy to RCs that use the energy to drive charge separation. RCs can also absorb a photon of light and undergo charge separation independent of LH1 and LH2. The RC contains a special pair of BChls that are able to absorb a photon of light to begin the cascade of electron transfers in the RC (discussed below). Therefore the antenna complexes increase the surface area and available wavelengths for light capture, allowing greater energy capture than the RC could independently accomplish. However, these antenna complexes are not a requirement for photosynthesis to take place.

**Reaction Center Structure**

The solution of the X-ray crystal structure of the *Blastochloris (Bcl.) viridis* RC by Michel and colleagues [12], followed by the subsequent solution of the X-ray crystal structure of the RC from *Rb. sphaeroides* [13, 14], opened a new era of relating structure to function of the
RC. This work will focus on the structure of the RC from *Rb. sphaeroides*, since it is more pertinent to the experiments presented here and there are significant structural differences between the *Bcl. viridis* and *Rb. sphaeroides* RCs.

The RC is an integral membrane protein and, depending on the genus/species of purple bacterium, there can be 3-4 subunits. The RC from *Rb. sphaeroides* is 102 kDa and has three subunits named for their apparent molecular weights in SDS poly acrylamide gel electrophoresis: L (light), M (medium), and H (heavy) [15]. The subunits actual molecular weights are: L = 31 kDa, M = 34 kDa , and H = 28 kDa [16-18]. The L and M subunits are each made up of five transmembrane α-helices that hold all the cofactors involved in the photochemistry of the RC and they are homologous to D1 and D2 in PS II [16]. The cofactors combined with the L and M subunits makeup the functional core of the protein for all purple bacteria. These cofactors include: a dimer of bacteriochlorophylls known as the special pair or primary donor (P) that is bound near the periplasmic surface, two monomers of bacteriochlorophyll (B), two monomers of bacteriopheophytin (I), two quinones (Q\textsubscript{A} and Q\textsubscript{B}) that are bound near the cytoplasmic surface, and (depending on the strain) sometimes a carotenoid. The carotenoid is not part of the functional core as it is not necessary for photosynthesis, but functions in a protective capacity [19]. Recent studies also suggest that it maybe necessary for RC assembly with the LH1 antenna complex [20]. A non-heme ferrous (Fe\textsuperscript{2+}) iron atom, ligated by four histidines (L190, L230, M219, M266 in *Rb. sphaeroides*) and Glu-M234, is bound between the two quinones (Figure 1).

The L and M subunits are similar, but not identical (33% of the amino acid sequences are identical) and form a membrane spanning dimer that has a pseudo-twofold axis through the membrane, which intersects the special pair of bacteriochlorophylls and the non-heme ferrous iron between the quinone binding sites [17].
Two branches, A and B, are formed by the cofactors and each branch is made up of chemically identical cofactors: one B, one I, and one quinone (branches are denoted by a cofactor with a subscript A or B). The $B_A$, $I_A$, and $Q_B$ cofactors are bound by the L subunit and $B_B$, $I_B$, and $Q_A$ cofactors are bound by the M subunit. However, electron transfer in the RC almost exclusively proceeds down the A branch starting from the P to $B_A$ to $I_A$ and then to $Q_A$ (the primary quinone), with $Q_A$ transferring its electron to the final electron acceptor, $Q_B$ (the secondary quinone) [21, 22]. As electron transfer only proceeds down the A branch and cofactors are chemically identical, the protein environment tunes the energetics to allow electron transfer down the A branch. Wakeham and co-workers took advantage of the homology of the L and M subunits and, with four mutations made a mutant RC that functions down the B branch [23].

The H subunit has one transmembrane helix that helps anchor it in place and a globular domain on the cytoplasmic side of the RC, making numerous contacts with the L and M subunits [14]. It is involved in proton transfer to $Q_B$, which is necessary for reduction of quinone to quinol [24]. Debus and coworkers showed that removal of the H-subunit significantly affected electron transfer to $Q_B$ [25]. However, other type II RCs exist without a H-subunit, e.g., *Roseiflexus castenholzii* [26].

**Reaction Center: Quinone and Electron Cycles**

Upon excitation with light, the excited singlet state of P ($P^*$) will transfer an electron to $B_A$. $B_A$ then subsequently reduces $I_A$. Next, $I_A$ reduces the primary quinone ($Q_A$) [27]. As quinone and quinol can readily diffuse in and out of the $Q_B$ site, if a quinone is bound in the proximal position of $Q_B$ (discussed below), $Q_A^-$ reduces $Q_B$ to a long-lived semiquinone ($Q_B^-$) [28,
The oxidized form of P (P\textsuperscript{+}) is reduced back to its uncharged state by cytochrome \(c_2\) to allow the reactions to be repeated. If no additional actinic light is added at this point, the charge separated state can be stable for minutes. With another flash of light, a 2\textsuperscript{nd} electron transfers from P\textsuperscript{*} through the A branch to Q\textsubscript{A}. As the second electron is transferred to doubly reduce Q\textsubscript{B}, two protons are taken up from the cytoplasm and Q\textsubscript{B} is reduced to quinol (QH\textsubscript{2}). QH\textsubscript{2} can readily diffuse out of the RC and into the membrane where the cytochrome bc\textsubscript{1} complex is able to oxidize QH\textsubscript{2} to quinone and return it to the membrane pool. The oxidized quinone can now return to Q\textsubscript{B} in the RC to be reduced again, completing the quinone/quinol cycle. During oxidation of QH\textsubscript{2}, the cytochrome bc\textsubscript{1} complex reduces oxidized cytochrome c\textsubscript{2}, which completes the cycling of electrons back to the RC [30]. The complete cycling of electrons and protons can be completed by chromatophores, which are sections of membranes that fold onto themselves creating discrete vesicle after the cell membrane has been disrupted. These chromatophores contain: LH1, LH2, RC, bc\textsubscript{1} complex, cytochrome c\textsubscript{2}, ATP synthase and other components in a membrane that can repeatedly convert light to chemical energy [31-33].

The entire process of transfer of an electron from P\textsuperscript{*} to Q\textsubscript{B} is completed in approximately 100 \(\mu\)sec. The 2\textsuperscript{nd} electron transfer and reduction of Q\textsubscript{B} to quinol is also completed in 0.1-1 ms depending on pH [34]. Figure 2 shows the complete turnover of reactions for the bacterial RC.

**Structure of the Q\textsubscript{A} and Q\textsubscript{B} sites in *Rb. sphaeroides***

The Q\textsubscript{A} and Q\textsubscript{B} binding sites are structurally homologous, but have different functions. *Rb. sphaeroides* binds a chemically identical quinone in both sites, where other RCs may sometimes have different quinones for each site, e.g., *Bcl viridis*. Both quinones are held in place by a helix-loop-helix motif between the D and E helices of the M subunit for Q\textsubscript{A} and the D and E
helices of the L subunit for Q$_b$ [14, 35]. The Q$_a$ site is made up of mostly non-polar amino acids. The Q$_b$ site, however, has numerous polar and even acidic residues within van der Waals contact of the quinone. The difference in polar residues is representative of the different functions between Q$_a$ and Q$_b$, specifically that Q$_b$ can be doubly reduced to quinol while Q$_a$ cannot. To accomplish this, Q$_b$ needs to have access to polar and acidic residues, specifically Asp-L210, Glu-L212, Asp-L213, and Ser-L223, for delivery of protons [36]. The polar difference also affects the dielectric environment at each site, which influences the $E_m$ and energetics of the two electron acceptors. Since quinone and quinol can readily diffuse in and out of the Q$_b$ site, competitive inhibition, e.g., with terbutryn, at Q$_b$ is possible [37]. The Q$_a$ quinone is fixed and cannot be exchanged under native conditions. Therefore, Q$_a$ cannot undergo competitive inhibition. Q$_a$ can only be singly reduced to the anionic semiquinone, while Q$_b$ can be double reduced.

In *Rb. sphaeroides*, Q$_a$ and Q$_b$ are both ubiquinone-10 (ubiquinone with a tail of 10 isoprene units. Abbreviated: UQ-10) and Q$_a$ binds UQ-10 with a $k_d$ of approximately 190 nM, while Q$_b$ binds UQ-10 with a $k_d$ of approximately 1.7 μM [38]. The head groups of both quinones are held in place by hydrogen bonds (H-bond) between the protein and the carbonyl oxygens of the quinones and by van der Waals interactions with the amino acids that make up the pockets for the quinones. Q$_a$ has a H-bond between the C1$^1$ carbonyl (C=O) of UQ-10 and the M260 peptide nitrogen, with a heavy atom distance of 2.79±0.09 Å, and a second H-bond between the C4 carbonyl of UQ-10 and N$_\delta$ of His-M219, with a heavy atom distance of 2.80±0.15 Å [39]. Residues Met-M218, His-M219, Thr-M222, Ala-M248, Ala-M249, Trp-

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1 There are two numbering conventions for the quinone head group. Here I use the numbering format based on benzoquinone as the parent molecule with the C1 carbonyl located next to the isoprene chain that is attached to C6. The other is based on toluene as the parent compound and is generally used with X-ray crystal structures.
M252, Asn-M259, Ala-M260, Thr-M261, and Ile-M265 are in van der Waals contact with the head group of QA, which gives the pocket an extremely high affinity for UQ-10 (Figure 3). Ile-M265 is of particular interest because it points directly at the head group of UQ-10 and has numerous van der Waals contacts with the quinone (discussed below), but is not directly involved in any of the H-bonds keeping UQ-10 in place.

Quinones can take two different positions in QB, the distal and proximal positions. The quinone is as close to the iron atom as possible when it is in the proximal position. To reach the distal position, the quinone head group must perform a 180° propeller-twist and move 5 Å from the proximal position as if it was diffusing out of the RC. The proximal position is believed to be the position in which QB can be reduced as it was shown to be the primary position for crystals frozen under illumination, in the charge separated state (Figure 4). The distal position is also farther from QA making electron transfer to the distal position significantly slower and therefore less likely. Further, the quinone in the distal position is only stabilized by one H-bond between the backbone amide of Ile-L224 and C4 carbonyl oxygen of the quinone [40]. In crystals frozen in the dark-adapted state, X-ray crystal structures showed QB to be primarily in the distal position. The quinone in the proximal position of QB is held in place by H-bonds between the C4 C=O of UQ-10 and the Nδ of His-L190 side chain (2.69±0.23 Å), and between the C1 C=O of UQ-10 and the hydroxyl of Ser-L223 (2.81±0.25 Å) and the peptide nitrogens of Ile-L224 (2.91±0.24 Å) and Gly-L225 (3.09±0.16 Å). Residues Leu-L189, His-L190, Leu-L193, Glu-L212, Asp-L213, Ser-L223, Ile-L224, Gly-L225, Thr-L226, and Ile-L229 are within van der Waals distance of the proximal QB site [39]. The movement of the quinone from the distal to the proximal position for reduction is suspected to be one explanation for the conformational gating hypothesis of the 1st electron transfer.
Chapter three and four will present the X-ray crystal structures from the polar mutants (Ser, Thr, Asn, and Gln) for Ile-M265 and discuss the structural changes around the $Q_A$ site.

**Kinetics:**

Many of the cofactors in the RC are spectroscopically active in the ultraviolet, visible, and near infrared spectrums. This has made it relatively easy to study the kinetics and energetics of the RC photochemistry events in great depth. In addition to monitoring the extremely fast forward reactions, it is also possible to monitor charge recombination from $P^+Q_A^-$ or $P^+Q_AQ_B^-$, which happens on the much slower time scale of milliseconds to seconds. This is referred to as dark relaxation or charge recombination. In the absence of $Q_B$ (1Q RCs) the back reaction rate ($k_p^A$) is $\approx 10 \text{ s}^{-1}$ in WT RCs [41]. In the presence of $Q_B$ (2Q RCs) the back reaction rate ($k_p^B$) is $\approx 1 \text{ s}^{-1}$ in WT RCs [42].

The rate of electron transfer in the RC is affected by many factors including the route an electron travels (tunneling vs. thermal), geometric orientation of donor/acceptor pairs, distance between donor/acceptor pairs, and electrostatic environment of the donor/acceptor pairs, including long range electrostatic effects. The kinetics are also different depending on other variables, including the availability of $Q_B$ as an electron acceptor, the type of quinone in $Q_A$ and/or $Q_B$ sites, the pH of the buffer in which experiments are being performed, and the environment around the RC (chromatophore, detergent or nano disk).

When the $Q_B$ acceptor quinone is absent or blocked by a competitive inhibitor, such as terbutryn or stigmatellin, $Q_B^-$ cannot form [37, 43, 44]. Therefore, charge recombination occurs from $Q_A^-$. In wild type RCs from *Rb. sphaeroides* this occurs by direct electron transfer (tunneling) to $P^+$ ($k_p^A$). When a low potential quinone, such as anthraquinone (AQ), is substituted
for $Q_A$ the charge recombination from $Q_A^-$ can recombine via the faster thermally activated route through $I_A$ [45].

In 2Q RCs, $P^*Q_AQ_B^-$ can recombine through $Q_A^-$ ($k_{BA^P}$) or by the much slower route of direct tunneling to $P^*$, $k_{BP}$ (dashed line in Figure 5). The direct route from $Q_B^-$ to $P^*$ makes up less than 5% of the charge recombination in wild type (WT) RCs from $Rb.\ sphaeroides$ [42]. However, it becomes more apparent in mutant RCs that have a relatively slow $Q_B^-$ back reaction such as M265IT and L213DN due to the increased midpoint potential difference ($\Delta E_m$) between $Q_A/Q_A^-$ and $Q_B/Q_B^-$ [46, 47].

Once $Q_B$ is singly reduced, the electron is in equilibrium between $Q_A^-Q_B$ and $Q_AQ_B^-$ ($Q_A^- Q_B \leftrightarrow Q_AQ_B^-$), and quantitated by the first electron-transfer equilibrium constant ($K_{AB}$). $K_{AB}$ is estimated to be 15-20 for $Rb.\ sphaeroides$ isolated RCs [42, 48]. As equilibrium constants cannot be directly measured, $K_{AB}$ can be estimated from the ratio of the rates of $Q_A^-$ and $Q_B^-$ back reactions: $K_{AB} = k_p^A / k_p^B - 1$. $K_{AB}$ is an approximation of the $\Delta E_m$ between $Q_A/Q_A^-$ and $Q_B/Q_B^-$. This approximation assumes that the direct tunneling route from $Q_B^-$ is not a significant contributor to charge recombination. When direct tunneling from $Q_B^-$ to $P^*$ becomes a significant contributor and $K_{AB}$ becomes significantly large, the ratio of the $Q_A$ and $Q_B$ back reactions is no longer an accurate estimate for $K_{AB}$.

Rate of charge recombination is also pH dependent [29]. The $Q_A^-$ decay rate is pH independent below pH 6 and weakly pH dependent above pH 6. $P^*Q_B^-$ charge recombination is more pH sensitive than $P^*Q_A^-$ charge recombination, being strongly pH dependent below pH 6.0 and above pH 9.0. In the pH dependent regions, the charge recombination rate increases with increasing pH for both $Q_A$ and $Q_B$ charge recombinations [42]. $P^*Q_B^-$ recombination is more affected by a broad range of pH, reflecting the large number of ionizable residues around the $Q_B$
site. Assuming a majority of the charge recombination occurs through $Q_A^-$, the pH dependence of $Q_B^-$ charge recombination will depend on the pH dependence of $K_{AB}$.

The rate of the forward first electron transfer ($Q_A Q_B \rightarrow Q_A Q_B^-; k^{(f)}_{AB}$) is approximately $10^4$ s$^{-1}$ at pH 7 in WT RCs from *Rb. sphaeroides* [49, 50]. The rate of a typical electron transfer is dependent on the free energy for electron transfer according to Marcus theory [51]. However, the rate of the first electron transfer in isolated reaction centers is not dependent on the free energy difference (driving force) between $Q_A/Q_A^-$ and $Q_B/Q_B^-$ [52]. This was shown by substituting quinones with different redox potentials in the $Q_A$ site and then studying how the rate of 1st electron transfer varied under these different driving forces. Graige and coworkers found no change in the 1st electron transfer rate as they varied the redox potential of the quinones in $Q_A$. They concluded that conformational gating was the rate limiting step and that as long as the inherent electron transfer rate was faster than the conformational gating step, increasing the $\Delta G$ for electron transfer would not increase the rate.

The second electron transfer, $(k^{(2)}_{AB}; Q_A Q_B \rightarrow Q_A Q_B H_2)$, has a rate of approximately $10^3$ s$^{-1}$ at pH 7 for WT. The second electron transfer is coupled to the uptake of the 1st proton and the second proton uptake rapidly occurs following the first proton uptake [53]. $k^{(1)}_{AB}$ is pH independent up to pH 9 but becomes slower at higher pH with an apparent $pK_a$ of approximately 9.5. $k^{(2)}_{AB}$ on the other hand is pH dependent through all pH values, decreasing in rate as pH increases.

Chapters three and four will give a detailed analysis of the kinetics of His-tagged isolated RCs from WT and the Ser, Thr, Asn, and Gln mutants for Ile-M265.

**Ubiquinone**
Bcl. viridis has a menaquinone and a ubiquinone in its Q_A and Q_B sites, respectively. The inherent potential difference between the two quinones is one factor that contributes to electron transfer from Q_A to Q_B being favorable [54]. In Rb. sphaeroides, nature has evolved a RC in which Q_A and Q_B are both the chemically identical UQ-10. This system requires that the protein provide an environment around each respective quinone site to tune their $E_m$, geometries, and distances to make reduction of Q_B by Q_A\(^-\) possible. This is achieved by the RC providing steric restraints on the quinone binding sites, controlling the electrostatic potential of the pockets, long distance electrostatic effects, and controlling the orientation of the methoxy groups of the UQ-10 head group.

The redox midpoint potential of a molecule is the potential at which it is equally willing to accept or donate an electron. In biologic systems, the midpoint potential difference between an electron donor and acceptor must be tuned so that electron transfer will be favorable. Since Q_A and Q_B are both UQ-10 in Rb. sphaeroides, the RC must tune the midpoint potential of Q_A/Q_A\(^-\) and Q_B/Q_B\(^-\) to make the formation of Q_B\(^-\) favorable. This makes the RC an excellent protein to study factors that control $E_m$ in biological systems.

Ubiquinone is unique among biological quinones in that it has two methoxy groups located next to each other in the 2 and 3 positions of a 1,4-benzoquinone ring. Due to their size and their ability to rotate freely at the oxygen-ring bond, the methoxy orientations can play a significant role in tuning the midpoint potential by either steric interactions or donation of electrons from the methoxy oxygens to the benzoquinone ring [55].

M265 Mutational Studies
Previous work in the Wraight lab probed mutations of Ile at position 265 of the M chain (Ile-M265), which is part of the binding domain for QA. Ile-M265 is in van der Waals contact with the following atoms of UQ-10 in the QA site: C3 methoxy group, C4 carbonyl and C5 methyl. Changing the non-polar Ile-M265 to a polar residue allows us to probe how changing the polarity or charge might affect the $E_m$ of QA. Since this position is within van der Waals contact of the C3 methoxy group of UQ-10, it was also hypothesized that changing the residue at M265 could change the orientation of the 3-methoxy on UQ-10 relative to the rest of the head group. If M265 were mutated to a larger residue, the QA pocket would be smaller and the 3-methoxy orientation could be sterically hindered. If the residue were smaller than Ile the pocket would have more room, allowing for the 3-methoxy to be able to rotate freely and orient itself to the most energetically stable orientation, which may not be the same as the position in WT. Mutating M265 to a polar or charged residue of similar size will change the dielectric environment at QA and this could also lead to a change in the $E_m$.

Takahashi et. al. [46] mutated Ile-M265 to the polar mutants Thr or Ser (M265IT and M265IS, respectively) to test if a hydroxyl (O-H) group in the QA site would change the $E_m$ of QA. A valine mutant (M265IV) was also made as a control to test if the smaller size of M265IT and M265IS affected the $E_m$ of QA, while not changing the polarity around QA. These small amino acids were chosen over others to avoid large structural changes to QA. Different quinones were substituted in the various mutants to test binding (naphthaquinones) and kinetic (anthroquinone) differences. The mutants had decreased binding affinities for quinones, in the QA site, with M265IS being the weakest binder and M265IV being the strongest. The M265IT and M265IS mutations led to a drop of the $E_m$ of QA/QA by approximately 100 mV and 80 mV, respectively. A similar drop in $E_m$ of QA/QA was confirmed with delayed fluorescence
measurements [56]. However, the M265IV mutation led to no significant change in $E_m$ at Q$_A$ from WT. When the low potential AQ was substituted in the three mutant Q$_A$ sites, the rates for all the mutants of the Q$_A$ back reactions were significantly faster. However, the rates of the polar mutants were 30-100 times faster than WT and the M265IV mutant. This ruled out methoxy orientation as the cause of change in $E_m$ because AQ does not have any methoxy groups.

Wells et. al. performed Fourier transform infrared spectroscopy (FTIR) on the polar M265 mutants and found an upshift of the 1601 cm$^{-1}$ band to 1603 cm$^{-1}$ in the polar mutants, compared to WT [57]. This band is assigned to the C4 C=O stretch and implied that there was a change in the H-bond between the quinone C4 C=O and N$_8$ of His-M219. Further, in silico structural energy minimizations indicated that the drop in $E_m$ might be caused by a lengthening of the H-bond between the C1 C=O of UQ-10 and the Ala-M260 peptide NH. This was suggested to arise from backbone perturbations due to a H-bond between the hydroxyl of the Thr or Ser at M265 and the backbone carbonyl of Thr-M261, and a steric interaction of the methyl group from the M265IT side chain and the Ala-M260 methyl side chain (Figure 6). The lengthening of the M260 H-bond would change the overall position of the quinone, leading to a shorter H-bond between the C4 C=O and N$_8$ of His-M219 [57].

However, pulsed EPR studies of the threonine mutant found no significant increase in the H-bond lengths to UQ-10 in the Q$_A$ site (E. Martin, S. Dikanov and C.A. Wraight unpublished). To examine the Wells et. al. hypothesis and further study if there was a structural reason for the results observed, or if the change was purely due to a change in $E_m$ at the Q$_A$ site from the mutation to a polar residue, I solved the X-ray crystal structures of M265IT and M265IS RCs. To further explore effects of different polar groups on the $E_m$ of Q$_A$, I mutated M265 to the novel
amide mutations of Asn and Gln (M265IN and M265IQ, respectively) and characterized the kinetics. To relate the structure to function, the X-ray crystal structures of M265IN and M265IQ were also solved.

References


The Bacterial Photosynthetic Reaction Center from *Rhodobacter sphaeroides*

**Figure 1:** The crystal structure of the bacterial RC from *Rb. sphaeroides* in a membrane with a quinone pool (1PCR) [58]. The three protein subunits, L, M, and H are represented by the cartoon drawing in orange, blue, and green, respectively. The cofactors are represented in the licorice drawing method with the primary donor (P), primary quinone (Q_A), and secondary quinone (Q_B) labeled. Horizontal lines represent the membrane/aqueous interface. The arrow from P to Q_A denotes the A branch of electron transfer and the arrow from Q_A to Q_B shows the reduction of Q_B by Q_A. Proton uptake and quinone/quinol exchange at the Q_B site are also represented by arrows at the Q_B site. Finally the reduction of the P^+ to P by the soluble cytochrome c_2 is shown on the periplasmic side of the RC. Combination of all of these reactions helps to give a cartoon representation of the turnover of quinones shown by the scheme in Figure 2.
Figure 2: Complete turnover of the bacterial photosynthetic reaction center. The lightning bolts represent a photon of light and $c_2$ is the reduced form of cytochrome $c_2$ and $c_2^+$ is the oxidized form. $Q$ represents the oxidized quinone and $QH_2$ is the fully reduced quinone (quinol).
Figure 3: The Qₐ site from the 1DV3 X-ray structure [59]. The arrows show the H-bonds between the backbone nitrogen of Ala-M260 and the C1 carbonyl of the quinone and between the Nₛ of His-M219 and the C4 carbonyl of the quinone. The lengths are in Ångstroms and are averages with standard deviation for all X-ray structures with greater than 2.80 Å resolution deposited prior to 2007 [39].
Figure 4: The Q₈ site from the 1DV3 X-ray structure [59]. The arrows show the H-bonds between the C₄ C=O of the quinone and the N₉ of His-L190, and between the C₁ C=O of the quinone and O-H of Ser-L223 and the backbone nitrogens of Ile-L224 and Gly-L225. The lengths are in Ångstroms and are averages with standard deviation for all X-ray structures with greater than 2.80 Å resolution deposited prior to 2007 [39].
Figure 5: Electron transfer pathways in the *Rb. sphaeroides* RC in the absence of an electron donor (first electron transfer and charge recombination). Dashed lines show thermal back reactions. Capital *K*s represent equilibrium constants and lower case *k*s represent rates.
Figure 6: Wells et. al. (2003) proposed M265IT structure. Using *in silico* modeling, the M265IT $Q_A$ site was shown to have the structure shown in this figure. The steric interaction between the methyl of M260 and the methyl of M265 was believed to cause the backbone of M260 and M259 to shift away from the quinone causing an increased H-bond to the C1 C=O and a shortened H-bond to the C4 C=O.
Chapter 2:
Materials and Methods

Abstract:

This chapter gives a detailed account of novel materials and methods used to complete this work that have not been published elsewhere. A complete detailed account of all non-novel experimental work is detailed in Appendix A.

Introduction:

Although *E. coli* are the most commonly used bacteria for studying prokaryotic proteins or systems, they lack all the biosynthesis pathways for the various cofactors and are therefore not yet suitable for expressing the bacterial photosynthetic reaction center (RC). For this reason I used the native species of bacteria that express the RC. Primarily these methods will focus on the purple non-sulfur bacteria *Rhodobacter sphaeroides* (*Rb. sphaeroides*).

This materials and methods section will discuss details of new experiments performed regarding purification of His-tagged RCs, crystallization of RCs, and X-ray crystallography data collection and analysis. Complete details for the mutagenesis, growth of wild type (WT) and mutant strains, purification of non-His-tagged RCs, quinone extraction and quinone substitution protocols, and flash spectroscopy kinetic studies, will be detailed in Appendix A.

His-tagged Reaction Center Purification:

His-tagged reaction centers can be purified in a significantly shorter time period (1-2 days) than non-His-tagged RCs (5-7 days). Many of the steps that must be performed with the non-His-tagged RCs can be omitted for His-tagged RCs, including the ammonium sulfate steps
which tend to cause significant losses of RCs. However, many of the initial steps are identical. See Appendix A for more details of non-His-tagged RC purification.

Lysing cells and Solubilization (all work should be performed on ice and intense light should be avoided where possible):

1. Frozen cell pellets are stored at -80 °C and it will take at least a few hours and preferably overnight to thaw the cell pellet. Add 150-200 mL of NET (100 mM NaCl, 0.5 mM EDTA, 10 mM Tris, pH 8.0) and a stir bar to the frozen cell pellet. Place the bottle on a stir plate in the cold room overnight. If there is a rush to thaw the cells, they can be thawed at room temperature on a stir plate and monitored regularly for thawing. Do not allow the pellet to thaw completely at room temperature. Once the pellet is mostly thawed it should be moved to the cold room to complete the thawing process. Before moving onto the next step, it is important to make sure the pellet is completely resuspended and that there are no solid clumps of cells floating around.

2. Centrifuge the cells at 7,000 x g, for 20 minutes at 4 °C. This step will wash the cell pellet of any unwanted material. After centrifugation, inspect the supernatant and the pellet. The supernatant should be clear. If it is not, pour off the supernatant and resuspend the cells. Centrifuge the cells again and continue to repeat until the supernatant is clear. When inspecting the pellet, if there is a large section of white at the core of the pellet, this will need to be removed to get an accurate cell mass. This large white mass is the byproduct of overgrowth from semi-aerobic growth. To remove the precipitate, follow the procedures described below. If there is no white pellet at the core, weigh the bottle with the cell pellet to determine the wet cell weight and proceed to step 3. Remember to weigh the empty bottle later and subtract the bottle weight from the bottle and cell pellet weight to get the actual cell weight.
Removal of precipitate: The white section in the pellet is precipitate from semi-aerobic growth. Fortunately it is significantly denser than the cells. To remove the precipitate, resuspend the pellet in 150 mL of NET and centrifuge the bottle at 1000 x g for 1 minute at 4 °C. Keep the supernatant, which should have your bacteria of interest. The pellet should be pure white. If there appear to be cells in the pellet, indicated by blue-green, green, or brown (depending on which strain of Rb. sphaeroides is being used), resuspend the pellet with 100 mL of NET and centrifuge the bottle again at 1000 x g for 1 minute at 4 °C. The supernatant from the second wash is combined with the first wash. Discard the white pellet. Step 2 is repeated to check for the white core pellet again. If there is any white core visible, repeat the removal of precipitate step until there is no white core pellet remaining.

3. After pelleting the cell only pellet, resuspend the pellet in NET with approximately 160 mL of buffer per 50 g of cells.

4. French press the mixture at 16,000 psi.

5. Centrifuge the crushed cells at 27,000 x g for 20 minutes to sediment unbroken cells and large debris. If a large pellet is present, resuspend in NET and repeat the French press step.

6. Put the RCs in a 37 °C bath for 30 minutes. Then put the beaker on a stir plate at room temp and add 30% stock LDAO, dropwise in the dark (cover beaker with foil) while stirring, to a final concentration of 1.0%

7. After 30 minutes of stirring, the solubilized RCs are poured into ultracentrifuge tubes and spun in the ultracentrifuge at 225,000 x g for 1.5-2 hours. Be sure tubes are full and top up with 1.0% LDAO in NET buffer. Any solubilized RCs that do not fit in the first spin of the ultracentrifuge are put in the 4 °C room, with continued stirring. Keep the supernant for nickel
column chromatography. When decanting off the supernatant, avoid any solids, no matter how small they are as they may clog the column during the next step.

8. Measure the near infrared absorbance spectrum of the sample, using 950 nm as a baseline to determine $A_{802}$. Then calculate the concentration of RCs by using the Beer-Lambert law as described in the appendix. The yield can vary significantly at this point, from anaerobically grown SMpHis to semi-aerobically grown mutants. The yield is usually 750 nmoles for SMpHis and 400 nmoles for semi-aerobically grown *Rb. sphaeroides* per 50 grams of cells.

**Nickel Column Chromatography:**

1. In the cold room (4°C), pour a nickel column using Qiagen Nickel-NTA resin. Wash the column with 4 column volumes of buffer 2 (10 mM Tris pH 7.8, 100 mM NaCl, 4 mM imidazole, 0.045% LDAO)

2. Load the supernatant from the ultracentrifuge onto the Ni-NTA resin column in the cold room. This step must be done very slowly, preferably overnight. Since the column is being loaded via a gravity system, it is important to make sure the column does not run dry. By running the tubing from the loading bottle to below the bottle of the column, the loading will automatically stop once it reaches a point in the tubing below the column. Collect the load flow-through and, once loading is completed, check for loss of RCs from the loading step (absorbance at 802 nm; $\varepsilon_{802} = 288$ mM$^{-1}$ cm$^{-1}$). If there is a significant loss of RCs from the load flow through (>15%), reload the flow through onto the column again.

3. Wash the loaded protein with 10 column volumes of Buffer 2 (≈ 500 ml). Collect the wash flow through and measure the absorbance at 802 nm to determine the loss of RCs from the wash step. If the loss is significant (>15%) this can be saved and reloaded onto a clean column.
4. Elute the RCs with Buffer 3 (10 mM Tris pH 7.8, 100 mM imidazole, 0.045% LDAO). The flow rate should be 1-2 mL per minute. Approximately 50-150 mL of Buffer 3 will be necessary to elute the RCs. Collect 5 mL fractions and measure the near infrared and ultraviolet absorbance of each fraction. Any fractions with a $A_{280}/A_{802}$ ratio of better than 1.5 are combined and concentrated to approximately 100 μM using a 30,000 molecular weight cutoff Centricon in the centrifuge at 2000 x g. These are then stored at -80 °C if they are to be used for kinetic studies or kept at 4 °C if they are to be used for crystallization. Any fractions with $A_{280}/A_{802}$ ratios above 1.5 are combined and either diluted and reloaded onto the Ni column or frozen at -80 °C for later purification.

5. The nickel column is washed with 3-4 column volumes of Buffer 2 once there no longer appears to be any color coming from the flow through of the column, and then left in the cold room for later use.

**Quinone Extraction and Substitution:**

Solutions:

Buffer QE: TL0.1:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>20 mM o-phenantroline</td>
<td>10 mM TRIS (pH 7.8)</td>
</tr>
<tr>
<td>(use 1 mM o-phenantroline</td>
<td>0.1 % LDAO</td>
</tr>
<tr>
<td>for Q₈ extraction only</td>
<td>0.05 mM EDTA</td>
</tr>
<tr>
<td>4.0 % LDAO</td>
<td>(Add 250 mM NaCl for elution)</td>
</tr>
<tr>
<td>1 mM 2-mercaptoethanol</td>
<td>10 mM TRIS (pH 7.8)</td>
</tr>
<tr>
<td>0.05 mM EDTA</td>
<td></td>
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</tbody>
</table>
**FPLC Protocols:**

1. Turn on the temperature controller and set to 27° C.

2. Equilibrate a DEAE-Sephacel™ column at room temperature with 2-3 volumes of TL0.1 or until the pH stabilizes at 7.8. Column volumes are usually 10-50 mL.

3. Load the purified RCs sample onto the column at a slow flow rate (≈ 0.25-1 ml/min). The sample can come straight from the fractions eluted from the Ni-NTA column without dialysis or concentration of the eluted RCs beforehand.

4. Once the sample is loaded, cover up the column with aluminum foil and wash it with ≈ 500 ml of Buffer QE that has been equilibrated to room temperature. This wash is performed at a rate of 1 mL per minute and therefore it is either performed overnight or started early in the morning. The flow through from the column should have a yellowish tinge that will become more and more yellow as the washing progresses. If the flow through becomes purple then bacteriopheophytin is being extracted with the quinones and the procedure should be stopped.

5. Wash the RCs with TL 0.1 until Abs$_{280}$ is close to 0.

6. The RCs are eluted from the column with 250 mM NaCl in TL0.1. If problems with sample purity arise, then the sample can be washed with a 100 mM NaCl solution in TL0.1 before the elution.

7. Eluted RCs should be collected in 5 ml fractions and either dialyzed overnight or concentrated in TL0.045 (10 mM TRIS (pH 7.8), 0.045% LDAO). The concentrated samples should be wrapped in aluminum foil to eliminate light exposure, and stored in the -80 °C freezer.
Losses of RCs from quinone extraction were often greater than 50%. RCs with no more than 15% residual $Q_A$ activity were used in 0 Q RC quinone substitution experiments.

Anthraquinone (AQ) was used as a low potential quinone for studying the influence of methoxy groups on the various M265 mutations. AQ was initially dissolved in ethanol to a concentration of 48 μM. The AQ was heated until the AQ was fully dissolved. The AQ was then placed in a microcentrifuge tube and the ethanol was evaporated by blowing nitrogen over the top of the tube. Concentrated RCs were added once the ethanol had been evaporated and the RCs were left to incubate at 0°C for 24 hours. The $Q_A$ back reaction kinetics of AQ RC were measured on the flash spectrophotometer.

**X-ray Crystallography:**

Crystallization, X-ray diffraction and Structure Solution

Crystallization protocols were based on those of Buchanan et al. and Pokkuluri et al. [1, 2]. Initially non-His-tagged RCs were used for crystallization trials, but His-tagged RCs were found to be much easier to purify in large quantities, higher purities, and in a shorter time. Therefore all crystal structures came from His-tagged RCs. RCs that crystallized were never frozen after the French press step during RC purification. The RCs were purified to a $A_{280}/A_{802}$ ratio of 1.2-1.5 and initially concentrated to approximately 100 μM concentration. The mutants M265IS and M265IT were each buffer exchanged to the crystallization buffer (10 mM Tris pH 7.8, 10 μM EDTA, 280 mM NaCl, 0.045% LDAO) using dialysis. The dialysis buffer was changed twice over the course of 12 hours at 4°C. The buffer for M265IN and M265IQ was exchanged by repeated Centricon concentration and dilution into the crystallization buffer. The
RCs were diluted three times to ensure complete exchange to the crystallization buffer conditions. After buffer exchange, all RCs were treated identically.

With the RCs in the crystallization buffer, the concentration of the RCs was rechecked and they were concentrated to a final concentration of 35-65 mg/mL. The goal was to concentrate the RCs to 32 mg/mL or higher, but some were concentrated much higher than planned. This did not seem to alter the ability for quality diffracting crystals to be grown. The RCs were aliquoted and diluted to 32, 30, 28, 26, 24, and 22 mg/mL. A screen was then performed with a 1:1 mixture with one of the following crystallization solutions:

A. 1.6 M potassium phosphate, pH 7.5
   6.4 % 1,2,3-heptanetriol
   4.0 % dioxane
   0.045 % LDAO

B. 1.6 M potassium phosphate, pH 7.5
   7.2 % 1,2,3-heptanetriol
   4.0 % dioxane
   0.045 % LDAO

C. 1.6 M potassium phosphate, pH 7.5
   8.0 % 1,2,3-heptanetriol
   4.0 % dioxane
   0.045 % LDAO

This yielded a final concentration of 16, 15, 14, 13, 12, and 11 mg/mL of RCs with a reduction of the concentration of the original crystallization solutions by half (e.g., A: 0.8 M potassium phosphate pH 7.5, 3.2% 1,2,3-heptanetriol, 2.0% dioxane). The LDAO concentration was
unchanged because the crystallization buffer also contained 0.045% LDAO. The crystallization solution and RCs were slowly mixed by adding the crystallization solution to the RCs to minimize precipitation of the RCs. Each set of conditions was run in triplicate (e.g., A-11 was set 3 times). The sitting drop vapor diffusion screens were set with 25 µL sitting drops and equilibrated against reservoirs filled with 1 mL of 1.6 M potassium phosphate, pH 7.5. The 24 well sitting drop plates were purchased from Hampton Research. The wells were sealed with clear packing tape and stored in the dark at 10-20 °C for up to 9 months.

Crystals grew in various conditions starting around 3-4 weeks. Some wells only grew crystals after 3-4 months. Many wells did not grow crystals at all, even if another well under the same exact condition had crystals. Precipitate was visible almost immediately in most of the wells. Crystals varied in shape, with some forming needles and others forming hexagonal columns. Crystals that grew were mounted on Hampton Research magnetic loops ranging from 0.1 mm-1.0 mm in loop size. Crystals varied in size from 0.1 mm-1.5 mm. The best diffraction was obtained with crystals grown from 12-14 mg/ml RCs in crystallization buffer. Crystals were cryoprotected using 28% ethylene glycol [3]. The crystals were placed into the 28 % ethylene glycol for at least 1-2 minutes. Often the crystals had a film of residual precipitate and crystallization solution, referred to as a skin. The skin was removed as best as possible while manipulating the crystals in the cryoprotectant. Crystals were then plunged into liquid nitrogen and stored in liquid nitrogen until they were ready for X-ray diffraction.

Crystals were shot with X-rays at the Advanced Photon Source at Argonne National Laboratories, at beam lines LS-CAT 21-ID D, F, and G at, at a temperature of 100 K kept stable by a cold nitrogen gas stream. Crystals were initially exposed to a 1 second burst of highly columnated X-rays. If the crystal diffracted to better than 3.5 Å on the first shot, then data was
collected on the rest of the crystal by rotating the crystal 0.75-1.00 degrees with 1 second exposures of X-rays. Detector distance was adjusted to maximize the high resolution data points collected and increase the resolution of points that were of lower resolution. Data was collected for at least 90°, but usually up to 180-270° to maximize data and completeness. Data collection was stopped early if the diffraction resolution significantly declined (> 3.5 Å). Data collection was also stopped if the crystal loop bent and/or failed, causing the data collection on these crystals to be difficult. If the crystal did not diffract to at least 3.5 Å resolution on the first X-ray blast, then a second X-ray exposure would be taken after a 90° rotation of the crystal. If this second exposure was still not of high enough resolution, and the crystal was of significant size (>0.5 mm), the beam focus was moved to another location and re-exposed. If this other location did not diffract well or the crystal was too small to sample another location, then the crystal was discarded.

Mosflm was used to examine diffraction frames and to confirm detector distance for analysis [4]. Diffractions were indexed, integrated and scaled using XDS [5]. XDS was rerun multiple times to maximize the highest resolution shell with a frequency of merit (FOM) of 80% or better, Rmerge of 80% or better, and a σ (signal to noise) of 2.0 or higher. Individual indexing of frames were examined to maximize the pixel count per image (>20,000) with a target of at least 1,000 strong signal points, if possible. Frames that did not meet the pixel and strong signal points criteria were excluded if there were enough high quality frames. Structures were solved using molecular replacement, with 1PCR [6] as the model, with Refmac5, and refined using Refmac5, Arpsolvent, and Phenix [7, 8]. Refmac5 and Arpsolvent are both part of the CCP4 crystallography suite. Chains were manually walked in Coot to check that the crystal structure
model fit the electron diffraction data [9]. Sections of the model that did not fit into diffraction data were deleted. Any mutations were also entered using Coot and then refined using Refmac5.

To have an unbiased comparison for any changes in the Q_A site, heavy atom distances between the quinone or protein and select nearby atoms were measured from all previous crystal structures of Rb. sphaeroides RCs available in the RCSB Protein Data Bank with better than 2.80 Å resolution. The list of crystal structures used in these calculations is given in Table 1. The mean and standard deviation from these structures is referred to as Xray-avg.

References:
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<tr>
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**Table 1:** List of crystal structures used for calculation of Xray-avg.
Chapter 3:
Structure and Function Studies of M265IS and M265IT

ABSTRACT:

Previous work showed that mutation of the nonpolar Ile at M265, in wild type (WT), which lies within van der Waals contact to QA, to the polar hydroxyl (O-H) mutants of Ser (M265IS) and Thr (M265IT) showed a drop in the in situ $E_m$ of QA by 85 and 100 mV, respectively (Takahashi, E., Wells, T. A. & Wraight, C. A. (2001), Biochemistry. 40, 1020-8.). In repeating Takahashi et al.’s kinetic work, it was discovered that there are two separate components for the QA$^-$ back reaction not previously recognized. The structures of the two mutants were solved using X-ray crystallography and the orientation of the M265 O-H, relative to the quinone, for the two mutants differ by 66°. The M265IS O-H is located in a position where four potential hydrogen bonds (H-bonds) are present, while the M265IT O-H is positioned where the O-H has only one potential H-bond. QA in M265IS has an additional H-bond, not present in WT, between the 2-methoxy of QA and the backbone nitrogen of M249, which maybe necessary to stabilize the quinone due to the increase in the size of the quinone binding pocket. For both hydroxyl mutants the H-bond to the C1 carbonyl of QA was significantly shorter than Xray-avg (the average of all atomic distances from currently deposited RC X-ray structures with resolution better than 2.80 Å) while only the H-bond to the C4 carbonyl of QA from M265IT was significantly shorter.
**Introduction:**

Electron transfer (ET) reactions are the basis of biological redox reactions. The bacterial photosynthetic reaction center (RC) has been the protein of choice for studying biological ET due to the numerous ET events between the RC’s many cofactors. Probing these reactions by mutating residues, substituting non-native cofactors, and changing solvent properties (pH, salt concentration, detergent concentration, temperature, etc.) gives insight into what controls the ET reactions and therefore redox properties of the system [1].

The functional core of RCs from purple photosynthetic bacteria is made up of two similar, but not identical subunits: L and M. A series of cofactors is contained within the functional core: 1 dimer of bacteriochlorophylls, 2 monomers of bacteriochlorophylls, 2 bacteriopheophytins, 2 quinones and 1 structural Fe atom [2].

The primary donor (P), a dimer of bacteriochlorophylls, in the RC is initially excited by a photon of light to its singlet state. The excited primary donor (P\(^\circ\)) quickly donates (200 ps) an electron to the primary quinone (Q\(_A\)), which subsequently reduces the secondary quinone (Q\(_B\)) in about 100 μs (Figure 5 from Chapter 1). Following reduction of the oxidized P (P\(^+\)) by cytochrome c\(_2\) and excitation of P by another photon, the cycle is able to repeat. With the uptake of two protons this ultimately leads to the reduction of Q\(_B\) from quinone to quinol. This quinol can then diffuse out and be replaced by an oxidized quinone so that the cycle may repeat. The quinol can then be used as energy currency for the cell via other proteins such as the bc\(_1\) complex [3].

In *Rhodobacter (Rb.) sphaeroides*, Q\(_A\) and Q\(_B\) are both ubiquinone with a 10 isoprene unit tail (Q-10). Since Q\(_A\) and Q\(_B\) are chemically identical the protein must tune the midpoint potentials (E\(_m\)) of the quinones to allow Q\(_A\)\(^-\) to act as a reductant for Q\(_B\) and Q\(_B\)\(^-\). Three major
factors that can control the $E_m$ of $Q_A$ are (de)stabilization of the semiquinone or the quinone through changes in electrostatics, alternate orientations of the Q-10 methoxy groups, and long-range interactions or global influences [1]. The major focus of this work will be $Q_A$.

The quinones that occupy $Q_A$ and $Q_B$ are held in place by van der Waals contacts and H-bonds. $Q_A$ has a H-bond between the C1 carbonyl (C=O) of Q-10 and the M260 peptide nitrogen, with a heavy atom distance of 2.79±0.09 Å, and a second H-bond between the C4 C=O of Q-10 and $N_δ$ of His-M219 side chain, with a heavy atom distance of 2.80±0.15 Å.² The head group of $Q_A$ is in van der Waals contact with residues Met-M218, His-M219, Thr-M222, Ala-M248, Ala-M249, Trp-M252, Asn-M259, Ala-M260, Thr-M261, and Ile-M265 [4]. The $Q_A$ site is relatively non-polar compared to the $Q_B$ site.

Ile-M265 is of particular interest because it points directly at the head group of Q-10 in the $Q_A$ site and is in van der Waals contact with the C3 methoxy group, the C4 carbonyl, and the C5 methyl of the Q-10 head group, but it is not directly involved in any of the H-bonds keeping Q-10 in place. Previous work by Takahashi et al. showed that mutation of this residue in the $Q_A$ site of the M subunit from the wild type (WT) Ile-M265 to the polar residues of Ser (mutant M265IS) or Thr (mutant M265IT) led to a drop in the $E_m$ of $Q_A$ by approximately 85 mV and 100 mV respectively [5]. However, mutation of the Ile to the similar sized non-polar residue Val did not lead to a significant change in the $E_m$. $Q_A$ was found to bind less tightly for the three M265 mutants with M265IS being the weakest. FTIR and in silico studies were also performed, suggesting that the H-bond between the C1 C=O of $Q_A$ and the M260 backbone N was longer in the polar mutants than in the WT [6].

² There are two numbering conventions for the quinone head group. This work uses the numbering format based on benzoquinone as the parent molecule with the C1 carbonyl located next to isoprene chain, which is attached to C6. The other is based on toluene as the parent compound.
Here I explore the mechanism behind the change in $E_m$ of the polar M265IS and M265IT mutants by solving the X-ray structure and examining the structure to function relationship of the $E_m$. I also present evidence partially supporting Wells’ hypothesis and report previously unrecognized biphasic kinetics of $Q_A^-$ back reaction.

**Results: Kinetics**

**Charge recombination in the absence of $Q_B$ ($P^+ Q_A^- \text{ charge recombination; } k_{pA}$):**

Charge recombination kinetics give insight into the energetics of the RC [7]. The presence of a His-tag did not significantly change $k_{pA}$, as supported by the comparison of $k_{pA}$ for His-WT and the previously published value for NT-WT [5] (Figure 7). $P^+ Q_A^-$ charge recombination for 6xHis-tagged M265IS and M265IT (16.5 s$^{-1}$ and 19.9 s$^{-1}$ at pH 8.0 respectively) were not significantly different from those previously reported for the non-his-tagged mutants. The 6xHis M265IT and M265IS each showed an additional component at all pH values measured that was not previously reported. The His-WT $Q_A$ back reaction was not divisible into two separate components. The pH dependence of the His-tagged RCs followed the same trend as previously reported, significantly accelerating charge recombination above pH 9.5.

**Biphasic nature of $k_{pA}$ for M265IS and M265IT:**

The $Q_A^-$ back reaction kinetics for the M265IS and M265IT mutants reported above were fit to two separate exponential decay components to get a well fit curve to the data. These two components (fast and slow) existed at all pH values measured (4-11) for the mutants, but the percentage of fast vs. slow varied greatly between mutants and when comparing some mutants to themselves at different pH values. The amplitude of the fast component for M265IS was
approximately 80% throughout the entire pH range measured. The fast component appeared to drop slightly in amplitude above pH 9.0, though it still made up greater than 70% of the total amplitude. At pH 7.0 M265IS had a $k_p^A$ of 15.6 s$^{-1}$, 2.9 s$^{-1}$, and 9.4 s$^{-1}$ for the fast component, slow component and weighted average of the two components, respectively (Figure 8). M265IT was roughly equally split between the fast and slow components over the entire pH range. At pH values above 8.5 the fast component increased to approximately 60% of the total amplitude for M265IT. This gave $k_p^A$ values of 50.0, 16.4, and 26.4 s$^{-1}$ for fast, slow, and weighted average components of M265IT respectively at pH 7.0 (Figure 9).

1st electron transfer ($Q_A^-Q_B^+\rightarrow Q_A^-Q_B^-; k_{AB}^{(1)}$):

All RCs studied showed 1st electron kinetics that followed the same trend (Figure 10). The kinetics were pH independent from pH 4.0-8.5 and pH dependent from pH 9.0-11, where the 1st electron transfer became significantly slower with increasing pH. His-WT 1st electron kinetics were not significantly different to NT-WT from pH 4.0 to 9.5, but above pH 9.5, His-WT was 40% slower than NT-WT. Both His-WT and NT-WT followed the same trend of decreasing rate with increasing pH in the pH dependent region. First electron kinetics below pH 7.0 and pH 7.5 for M265IT and M265IS respectively, were faster than NT-WT and His-WT by ~50%. From pH 7.0 for M265IT and 7.5 for M265IS until pH 9.5, the 1st electron transfer kinetics for both mutants was approximately the same as NT-WT. Above pH 9.5, the rates of both mutants were faster than NT-WT, but similar to His-WT. It is of note that M265IT and M265IS showed the same pH dependence trend as His-WT above pH 7.0, which markedly is different from that reported previously by Takahashi et al (2001) (Figure 10).
Charge recombination in the presence of $Q_B$ ($P^+Q_AQ_B → PQ_AQ_B$; $k_P^B$), Apparent 1st electron transfer equilibrium constant between $Q_A$ and $Q_B$ ($Q_AQ_B ←→ Q_AQ_B$), and 2nd electron transfer ($Q_AQ_B → Q_AQ_BH_2$; $k^{(2)}_{AB}$):

$k_P^B$, $K_{AB}$, and second electron transfer kinetics for His-WT and for the mutant RCs were not found to be significantly different from their previously published untagged equivalents (Figures 11, 12, and 13).

Results: X-ray Crystal Structures

M265IS and M265IT Structures and significant $Q_A$ site changes:

The crystals for both structures were tetragonal and both diffracted in the $P3_121$ space group, with unit cell dimensions of $a=b \approx 139.53 \pm 0.66$ Å and $c \approx 185.03 \pm 0.47$ Å (Table 2). The rms bond length deviation was at most 0.035 among the structures and the rms bond angle deviation was at most 3.196°. The number of Ramachandran violations varied from 31 to 42 in the structures. Table 3 lists atomic distances from $Q_A$ of each structure in comparison with Xray-avg distances. For purposes of comparison the 1DV3 structure was aligned with each of the mutants in VMD and the Ile-M265 has been left visible in the crystal structures shown in Figure 14 [8, 9]. Each crystal and the respective changes to $Q_A$ are reported below.

X-ray Crystal Structure of M265IS:

The structure of M265IS was solved to a resolution of 2.77 Å and refined to a R factor of 19.1% ($R_{free} = 22.7\%$) (Table 2). The H-bond between the C1 C=O of the quinone head group and the N of Ala-M260 was found to be significantly shorter at 2.66 Å vs. 2.78 ± 0.108 Å in
X-ray avg. The H-bond between the C4 C=O of the quinone head group and N$_\delta$ of His-M219 was longer at 2.94 Å vs 2.82 ± 0.133 Å in X-ray avg, but this was not significantly different (see discussion). The hydroxyl (O-H) of M265IS occupies a similar space to the $\gamma_2$ carbon of the M265 isoleucine side chain from the 1DV3 structure when the structures are aligned (Figure 14) [8]. In this position there are four potential H-bonds. These are between the M265 serine O-H and the backbone C=O of M262 (2.72 Å), the backbone nitrogen of M266 (3.04 Å), the N$_\delta$ of His-M266 (3.11 Å), and the backbone C=O of M261 (3.28 Å) (Figure 15). The crystal model also shows a potential H-bond between the 2-methoxy oxygen of the QA head group and the backbone nitrogen of M249 with a distance of 3.25 vs. 3.51 ± 0.195 Å in X-ray avg.

**X-ray Crystal Structure of M265IT:**

The structure of M265IT was solved to a resolution of 2.97 Å and refined to an R factor of 17.4% ($R_{free} = 19.4\%$) (Table 2). M265IT had both H-bonds between the quinone head group carbonyls and protein significantly shortened, with the C1 C=O H-bond at 2.54 Å vs. 2.78 ± 0.108 Å in X-ray avg and the C4 C=O H-bond at 2.60 Å vs 2.82 ± 0.133 Å in X-ray avg (Figure 14). The hydroxyl of the M265IT side chain is rotated away from M260, approximately 66° from the hydroxyl position of M265IS, and toward histidine M219, creating a weak potential H-bond between the Thr-M265 O-H and N$_\delta$ of M219 (3.39 Å). (Figure 16). The M265 hydroxyl also may have an internal H-bond with the backbone C=O (3.17 Å), although the angle is far less favorable. Finally, there may also be a weak H-bond between the backbone nitrogen of M249 and the oxygen from the 2-methoxy position on the quinone head group (3.49 Å vs. 3.51 ± 0.195 in X-ray avg). The side chain of His-M219 is tilted and shifted, which maybe partially responsible for the shortened H-bond between the C4 C=O of QA and N$_\delta$ of M219 (Figure 16).
The distance between the backbone C=O of Thr-M261 and the backbone N of M265 is 2.99 Å, not significantly changed from 3.03 of 1DV3.

**Discussion: Kinetics**

The addition of a single polar group at M265 in place of the non-polar Ile has a very large effect to the $E_m$ of $Q_A$. Based on the lowered $E_m$ of $Q_A$ and the weaker quinone binding at $Q_A$, the semiquinone in the $Q_A$ site must be destabilized for the M265IS and M265IT mutants [5]. Some of the possible causes of the destabilization of the semiquinone are a change in the polarity or dielectric of the $Q_A$ site due to the addition of the hydroxyl, structural changes and/or electron distribution in $Q_A$ due to new H-bonds formed, alternate orientations of the methoxy groups, or long-range interactions and global influences. To provide further insight, I looked to the X-ray crystal structures, which are discussed below.

**His-tag vs. Non-His-tagged:**

There are no significant differences between the kinetics of NT-WT RCs and the kinetics of the 7xHis His-WT RCs. For the mutants, only the two components in the $Q_A$ back reaction and the dependence of 1" electron transfer pH (see above) were significantly different when comparing between the 6xHis-tagged mutants and their untagged counterparts. These differences were attributed to a difference in analysis, discussed below. As I could not separate the $Q_A$ back reaction of His-tagged RCs into two separate components, I assumed that the two components are caused by the mutation of M265 rather than the addition of the His-tag. Due to the lack of unexplained significant differences in the kinetics between his-tagged and non-his-tagged RCs I
conclude that the RCs are not different and will be treated the same throughout the rest of this work.

**P\(^+\)Q\(_\lambda\) \(-\)** biphasic kinetics:

The biphasic kinetics of the Q\(_\lambda\) \(-\) back reaction must be a result of the mutations and not the his-tag as it was not possible to separate out two components from His-WT. The two components were likely not previously noticed because the data fits a one-component analysis quite well. When looking at NT-M265IS data from Takahashi et al 2001, it is quite similar to the fast component data for His-tagged M265IS (Figure 7). This is not surprising as the fast component makes up approximately 80% of the amplitude throughout the entire pH range measured for M265IS. The slow component could easily have been missed if only looking for the fast component or only measuring a limited amount of the decay of P\(^+\). It is also possible that the slow component was ignored as some baseline issue or failure of terbutryn because the rate is on the order of the Q\(_{hi}\) \(-\) back reaction for WT. A charge recombination that slow would not make sense from Q\(_\lambda\) \(-\) and would difficult to explain. Therefore I believe that whatever the reason, Takahashi and coworkers were correct in their treatment of using the fast component only for Q\(_\lambda\) \(-\) back reaction. Unfortunately this does not hint at what this extremely slow component is.

NT-M265IT from Takahashi et al 2001, on the other hand, fits the weighted average of the fast and slow components of the His-tagged M265IT quite nicely. As these are both approximately 50% throughout the pH range measured, it is not as easy to miss either of them in analysis. If a single component analysis was used, this is the same result that would have been drawn. This time however, the slow component for M265IT is similar to the fast component of M265IS, but the fast component of M265IT is extremely fast. It is approximately 7-8 times
faster than the back reaction of WT. It is possible that this fast component is the charge recombination from $Q_A^-$ and that the slower component is another charge recombination reaction. If this were the case, the difference would be an approximate drop of 150 mV rather than 100 mV. However, I cannot differentiate at this time what each component is.

It is not clear what the cause of the two components of the $Q_A^-$ back reaction for the two mutants are. I speculate that it could have to do with rotation of the hydroxyl group by rotation of the $\alpha$ and $\beta$ carbon bond of Ser and Thr. However, the rate of this rotation is on the wrong time scale and it would likely equilibrate quickly not allowing us to separate the two components. A more likely possibility is a slight movement of the quinone head group to a second stable position. Displacement would not need to be very far for the results to occur. However, the crystal structures of M265IS and M265IT do not hint at such a position. Another possibility is that the slow component could be terbutryn being reduced in the $Q_B$ site. This would also make sense due to M265IS having approximately 20% slow component and M265IT having 50% reduced with M265IT having a larger driving force. At the present time I can only speculate and do not have an explanation as to why there are two components with these mutants for the $Q_A^-$ back reaction.

**The first electron transfer ($Q_A^-Q_B \rightarrow Q_AQ_B^-$):**

The first electron transfer kinetics are known to be independent of the $E_m$ difference between $Q_A^-/Q_A$ and $Q_B^-/Q_B$ [4, 10]. Rather, electron transfer to $Q_B$ has been proposed to be controlled by the electrostatic potential generated in the $Q_B$ site by a cluster of acidic residues including Glu-L212 [7, 11-17]. The cluster of acidic residues has a pK of approximately 9.5 and the rate of 1st electron transfer is determined by the fraction of ionizable species in the protonated (neutral)
state, which, above the apparent pK, decreases in proportion to the pH [5, 18, 19]. There have been many structure-based electrostatic calculations performed that support the $Q_b$ acid cluster idea [20-23].

The acid cluster that has been proposed to control the rate of 1st electron transfer between $Q_A$ and $Q_B$ is in the local region of $Q_B$ and the previously published M265IS and M265IT pH dependence trends were surprising because the mutations in the $Q_A$ site caused such a significant change to the pK. These previously published results showed a lower pH dependent region for NT-M265IT and NT-M265IS from pH 7.0 to 9.5, with a second pH independent region (pH 10.0-11.0) [5]. However, the 1st electron transfers of the His-tagged M265 mutants showed the same pH dependence trends (4.0-8.5 pH independent and 9.0-11.0 pH dependent) as WT (figure 10). The only differences between M265IT and M265IS in Takahashi et al. (2001) and this work are the 6xHis-tags. After carefully repeating the experiments with NT-M265IT I did not see the pH dependence previously published (data not shown). A pH dependence trend similar to what was observed by Takahashi et al. became apparent when a two-component analysis was taken and only the slow component was used. Based on this, the different trends are based on how the data was analyzed, rather than the addition of the 6xHis-tag. I believe the data presented in this paper is correct, which is consistent with the mutations being in the $Q_A$ site and therefore not affecting the 1st electron transfer kinetics.

**Discussion: X-ray Crystal Structures**

The crystal structures are only a snapshot of likely the ground state, though there is some debate on this due to the exposure of X-rays that could form free radicals and alter the structure [24]. Using X-ray crystallography I am not able to determine the crystal structure of $Q_A$ in the
semiquinone state. Yet, the ground state structures still give hints as to important structural changes that could potentially be calculated using molecular dynamics or electrostatic calculations.

**M265IS:** Serine is the smallest side chain of the M265 polar mutants. M265IS has the weakest binding $Q_\Lambda$ site of the M265 mutants [5]. This is supported further by the crystal structure showing the hydroxyl group pointing away from the quinone head group of $Q_\Lambda$ and thus increasing the size of the binding pocket. The $\beta$, $\gamma_1$, $\gamma_2$, and $\delta$ carbons of the Ile side chain of M265 in WT are within van der Waals contact with C3 methoxy group, C4 carbonyl, and C5 methyl group of the quinone head group of $Q_\Lambda$. The Ser side chain only has the O-H and $\beta$ carbon within van der Waals contact with the C3 methoxy group of the quinone head group of $Q_\Lambda$. Due to the loss of van der Waals contacts, there is more room for the quinone to move and the average B-factor of the $Q_\Lambda$ head group of 45.37 in M265IS vs. 28.79 in 1DV3 hints that there maybe more fluctuation. However, previous studies have found that B-factor structure to structure comparisons do not correlate well, as the differences in structures are more attributable to crystal packing [25].

By reducing the number of van der Waals contacts and changing the electrostatic environment surrounding the quinone by adding a hydroxyl group, there are possibilities for changes to the quinone position of $Q_\Lambda$ relative to the protein. These lead to changes in the length of H-bonds between the C=O of the quinone and the protein and the possibility of new H-bonds between $Q_\Lambda$ and the protein when compared to WT. The M265IS crystal model shows a potential H-bond between the 2-methoxy oxygen of the $Q_\Lambda$ quinone head group and the backbone nitrogen of M249, with a distance of 3.25 vs. $3.51 \pm 0.195$ Å in Xray-avg, which may
help stabilize the quinone head group to compensate for the loss of the van der Waals contacts discussed above. It may also stabilize the orientation of the 2-methoxy group, which is known to affect the $E_m$ [26-28].

When the M265IS and 1DV3 structures are aligned, the oxygen of the hydroxyl from Ser-M265 occupies a position that overlaps with the $\gamma_2$ methyl of Ile-M265 in 1DV3 (Figure 15). Both a polar group and non-polar group structurally occupy a similar location when comparing the $\gamma_2$ methyl and O-H of M265 in the M265IS and WT crystal structures. This is an indication that this position is either extremely favorable for M265IS due to the additional H-bonds or sterically it is favored as it is present in both 1DV3 and M265IS. The 1DV3 structure shows the Ile-M265 $\gamma_2$ carbon is within 3.5 Å of four polar groups. In the M265IS structure, the hydroxyl of Ser-M265 is positioned near these same four polar groups so that the O-H has four potential H-bonds, making the M265 O-H position very favorable (Figure 15). These potential H-bonds will significantly stabilize the hydroxyl in this position. The H-bond between the Ser-M265 hydroxyl and the backbone C=O of M262 (2.72 Å) likely helps pull the backbone C=O of M261 closer, allowing it to form a H-bond as well (3.28 Å). These two hydrogen bonds appear to be responsible for pulling the backbone nitrogen of M260 closer to the quinone head group, leading to the shorter C1 C=O to M260 nitrogen H-bond (2.66 Å vs 2.78 Å in Xray-avg). A similar argument can be made for the lengthening of the H-bond between the C4 C=O of the quinone head group and the $N_\delta$ of His-M219. The serine hydroxyl in M265IS forms a potential H-bond with the $N_\delta$ of His-M266 (3.11 Å) and the backbone N of M266 (3.04 Å). These two potential H-bonds pull His-M266 slightly closer to M265, altering the interaction between the $\varepsilon$ nitrogen and the iron. This leads to a perturbation of the M219 histidine/iron interaction that causes the
imidazole side chain of His-M219 to tilt slightly and increase the C4 quinone H-bond to 2.94 Å vs. 2.82 ± 0.133 Å in Xray-avg.

The crystal structure of M265IS does not completely rule out a contribution of the methoxy dihedral angles to the change of $E_m$ of $Q_A$ as the crystal structure shows the methoxy dihedral angles to be 126.05° for 2-methoxy and -82.43° for the 3-methoxy. However, it is likely that this difference would be minimal as both dihedral angles not significantly different from the average angles reported by Wraight and Gunner of 139 ± 25° and -77 ± 8° [4].

These structural changes to the $Q_A$ site are the major contributors to the change of binding affinity of ubiquinone to $Q_A$. As mentioned above, the decrease in $E_m$ and lower affinity for quinones in $Q_A$ is caused by a destabilization of the semiquinone. There may also be a slight contribution from the methoxy dihedral angles, though this is a minor component of the total change in redox potential of $Q_A$ indicated by the anthraquinone substitution experiments previously performed. Changes in polarity and electrostatics in the $Q_A$ site of M265IS cannot be ruled out or estimated using these models alone and will need to be calculated using electrostatic calculations. It is difficult to predict long-range influences or global interactions and no significant distant structural changes were noted in the mutant crystal structure.

**M265IT:**

M265IT was previously shown to have a decreased binding affinity for quinones [5]. From the crystal model, this decreased binding affinity is at least partially caused by a loss of van der Waals contacts as with M265IS. The side chain of Ile-M265 is in van der Waals contact with the C3 methoxy group, C4 carbonyl, and C5 methyl group of the quinone head group of $Q_A$, but the side chain of Thr-M265 has lost the C5 methyl group contact (Figure 16). The δ carbon of
the side chain of Ile-M265 in WT caps the Q on the isoprene tail side of the quinone. The loss of this contact in M265IT increases the dissociation constant for Q in M265IT. I therefore conclude that the δ carbon of Ile-M265 is important for binding, but not critical for RC function.

The crystal structure of M265IT does not completely rule out the methoxy dihedral angle contribution as the crystal structure results shows the methoxy dihedral angles to be 118.17° for 2-methoxy and -83.54° for the 3-methoxy. These are very similar to those of M265IS and not significantly different from the WT-average angles reported by Wraight and Gunner of 139 ± 25° and -77 ± 8° [4]. My structures support the previous conclusion that the methoxy groups are not the major contributor to the change in E observed.

From the crystal structure of M265IT, it is clear that the side chain hydroxyl takes a different orientation to the hydroxyl of M265IS. Instead, the O-H of M265IT is pointed away from the M261 backbone carbonyl and towards the Nδ of His-M219. This orientation is favored not because of increased stability from H-bonds, but rather from decreased steric interaction. If the O-H of M265IT were in the same orientation as the O-H in M265IS, the Cγ of the M265 side chain would be in an extreme steric clash with the Cβ of Ala-M260 at a distance of approximately 2.14 Å. In the WT crystal structure, the Cγ of the M265 and the Cβ of Ala-M260 are 3.13 Å apart, which is already an unfavorable steric distance. The Cγ of the M265IT has an even greater steric interaction with the backbone C=O of M261 with a heavy atom distance of 2.77 Å. From a H-bond prespective, the O-H of M265IT has only one potential weak H-bond at 3.39 Å with the Nδ of His-M219 to help keep it in this orientation. The electron density of the M265IT side chain is well defined, indicating that even though there are unfavorable steric interactions in the crystal structure, this position is the dominant orientation. Another reason why the side chain of Thr-M265 is rotated and different from Ser-M265 is that Thr is larger. Ser
is smaller than Thr and is able to take on different orientations due to its smaller size. However, this does not explain why Ile is able to assume a structure that is closer to M265IS than M265IT, even though size wise Ile is closer to Thr.

There appears to be little to no change of the Qₐ quinone position in the M265IS crystal structure compared to 1DV3. The M265IT Qₐ position is also virtually identical to 1DV3 except that the quinone is tilted 9.7° reducing the distance between the C4 carbonyl of the quinone head group and the N₈ of His-M219 (2.60 Å vs 2.82 ± 0.133 Å). This shorter distance and tilting of the quinone may be a consequence of the potential H-bond formed between the hydroxyl of the Thr of M265 and N₈ of His-M219 (3.39 Å). The position of the O-H may also be stabilized by the potential internal H-bond between the O-H of M265 and the C=O (3.17 Å).

Although Qₐ of M265IT has a slightly higher affinity for quinones than M265IS, it has a larger drop in Eₘ of Qₐ/Qₐ⁻ (100 mV vs 85 mV). The larger drop in Eₘ is likely caused by the effects of the position and orientation of the hydroxyl of Thr-M265 to the Qₐ pocket. The weak H-bond between Thr-M265 and N₈ of His-M219 seems to aid in shortening the H-bond between the quinone C4 C=O and N₈ of His-M219. If this were a bifurcated H-bond, one would expect the C4 C=O H-bond to be lengthened and not shortened. However, if the O-H of M265 is acting as a donor to the N₈ of His-M219 it may increase the strength of the C4 C=O H-bond [29].

There may also be a small contribution from the methoxy dihedral angles, though this is probably a minor component of the change in redox potential of Qₐ as discussed above. The position and orientation of the O-H group from Thr-M265 is likely the largest contributing factor for the large change seen in redox potential of Qₐ.
Conclusions:

The X-ray structures give an excellent snapshot of the ground state of the M265IS and M265IT mutants. For these two mutants, the most significant contributions to the change in redox potential for $Q_A$ is the strength with which $Q_A$ is bound and change in the dielectric of the $Q_A$ site, which is partially controlled by the orientation of the O-H group of serine and threonine. The X-ray structures are useful when examining the quinone in the ground state, but not very useful when trying to compare the charge separated or semiquinone states. Other techniques specifically suited to studying charged states, such as EPR are needed to assess the semiquinone effects for these mutants. These ground state structures can be used as a model to extrapolate changes that may occur with the addition of a charge using in silico modeling techniques. This may further elucidate the exact mechanism behind changes in $E_m$ that occur.

References:

7. Shinkarev, V.P. and C.A. Wraight, Electron and Proton Transfer in the Acceptor Quinone Complex of Reaction Centers of Phototrophic Bacteria, in The Photosynthetic


Figures and Tables:

**Figure 7:** pH dependence of $k_{P^+}$ of M265 mutants

The figure shows the pH dependence of $k_{P^+}$ of M265 mutants. The data points represent the disappearance of $P^+$ at 430nm, in the presence of 50μM terbutryn to block $Q_b^-$ formation. NT-WT, NT-M265IS, and NT-M265IT data are here for comparison from Takahashi et al 2001. M265IS-fast comp only shows only the fast component kinetics. M265IT shows the weighted average of the two-component analysis.
Figure 8: Biphasicity of $k_p^A$ for M265IS. The top graph shows the slow vs fast component percentages at each pH value measured. The bottom graph shows the rate of the fast, slow, weighted average components over the entire pH range.
Figure 9: Biphasicity of $k_p^A$ for M265IT. The top graph shows the slow vs fast component percentages at each pH value measured. The bottom graph shows the rate of the fast, slow, weighted average components over the entire pH range.
Figure 10: pH dependence of 1st electron transfers in M265 mutants. NT-WT and NT-M265IT data are here for comparison from Takahashi et al 2001.
**Figure 11:** pH dependence of $P^+Q_AQ_B^-$ charge recombination monitored by the disappearance of $P^+$ at 430nm, in the presence of 20μM Q-10 to increase the likelihood of $Q_B^-$ being occupied by Q-10. NT-WT and NT-M265IT data are here for comparison from Takahashi et al 2001.
Figure 12: pH dependence of the apparent equilibrium constant between $Q_A Q_B$ and $Q_A Q_B^-$. NT-WT and NT-M265IT data are here for comparison from Takahashi et al 2001.
Figure 13: pH dependence of the rate of 2nd electron transfer in the M265 mutants. NT-WT and NT-M265IT data are here for comparison from Takahashi et al 2001.
<table>
<thead>
<tr>
<th>Mutant</th>
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<tr>
<td>PDB ID Code</td>
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<td>4H99</td>
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**Crystal Data**

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<td>Completeness (highest shell) (%)</td>
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**Refinement**

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<tr>
<td>R Free</td>
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<td>From Wilson Plot (B-value)</td>
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<td>rms bond angle deviation (°)</td>
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**Table 2:** This table lists the summary of results from the analysis of the X-ray structures of the M265IS and M265iT mutants.
Table 3: A list of selected distances from the mutant crystal structures, with a comparison of Xray-avg distances. All distances are in the QA site of the RC. Numbers in red are significantly different compared to Xray-avg.

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<td>4.92</td>
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Figure 14: The $Q_x$ site in the X-ray crystal structures of the M265IS and M265IT are shown in this VMD representation. Heavy atom interactions of interest are indicated with arrows between them and the distance between the two heavy atoms is given in ångstroms. Numbers in parentheses below a distance are the Xray-avg distance. Distances are either black for not significantly different or red for significantly different from Xray-avg. Distances without values in parentheses below did not have corresponding values from Xray-avg. The red is used to highlight the short distances, which may either be H-bonds or steric clashes. The Ile-M265 amino acid from the 1DV3 structure is present in gray for comparison. The structures were aligned using SEQ in VMD [9].
Figure 15: The X-ray structure of M265IS. Arrows indicate potential H-bonds formed between the O-H from the Ser-M265 and either backbone C=O (M261 and M262) (left panel), or backbone N and N_{\delta} of His-M219 (M266) (right panel).
Figure 16: The Q site of M265IT with a comparison to the 1DV3 structure. Numbers in parentheses are from Xray-avg. The 1DV3 structure was aligned and is shown in gray for comparison.
Chapter 4:

Structure and Function Studies of M265IN and M265IQ

Abstract:

A significant drop in the $E_m$ of $Q_A$ was previously found with mutation of Ile-M265 to the polar Ser and Thr mutants. As both of these are hydroxyl mutants, the hypothesis was proposed that placing a different polar group at the M265 position would also decrease the $E_m$ of $Q_A$. The Asn and Gln amide mutants for M265, M265IN and M265IQ, respectively, were cloned and showed quite different kinetics from one another. M265IN showed kinetics quite similar to the hydroxyl mutants, but M265IQ showed kinetics that did not significantly alter the $E_m$ of $Q_A$ from WT. It was further found by kinetic spectroscopy that $Q_A$ is displaced approximately 50% of the time in M265IQ. The X-ray crystal structures for M265IN and M265IQ were also solved. The M265IN structure showed the M265 side chain amide interacting with the C4 carbonyl of $Q_A$ and with the N$_\delta$ of His-M219. The M265IQ Gln side chain showed two conformations. Conformation A shows the M265 amide side chain interacting with the N$_\delta$ of His-M219 and with the C4 C=O of the quinone. This leads to a significantly shorter C1 C=O H-bond. Conformation B shows the Gln side chain in a position where it significantly sterically clashes with the quinone, alluding that a bound quinone in this conformation is highly unlikely. The occupancy of the quinone by electron density is also found to be approximately 50%, supporting the kinetic data and further strengthening the idea that the quinone is likely displaced in Conformation B.

Introduction:

In the *Rhodobacter sphaeroides* (*Rb. sphaeroides*) RC, the final two electron acceptors are both ubiquinone-10 (UQ-10). The second to last and last electron acceptors are known as the
primary quinone ($Q_\lambda$) and secondary quinone ($Q_B$), respectively. As these two quinones are both the same molecule chemically, the protein must tune the midpoint potential ($E_m$) of each quinone to make the reduction of $Q_B$ and the semiquinone of $Q_B$ ($Q_B^-$) favorable by the semiquinone of $Q_\lambda$ ($Q_\lambda^-$). Numerous variables affect the $E_m$ of $Q_\lambda$ and $Q_B$ including the local dielectric environment, binding affinity, pH, and long-range or global interactions.

Previous work by Takahashi and coworkers found that mutation of Ile-M265 to the polar mutants Ser and Thr, M265IS and M265IT, respectively, reduced the $E_m$ of $Q_\lambda$ by approximately 100 mV [1]. To correlate the structure to function relationship, Mattis and Wraight (manuscript in preparation), solved the X-ray structures for M265IS and M265IT. The structures showed that the hydroxyl groups were rotated in different directions in the two mutants, but this did not seem to alter the $E_m$ significantly between the two hydroxyl mutants. This indicated that simply having the polar group in the M265 position altered the $E_m$ much more than the direction of the dipole of the added polar group. Here I further explore how adding a different polar group to M265 could further elucidate what factors are most important in tuning the $E_m$ of $Q_\lambda$. The amide mutants at M265, Asn and Gln, respectively known as M265IN and M265IQ, were cloned and the electron transfer kinetics were characterized in these novel mutants. The X-ray structures of both of the amide mutants were then solved and structure to function interpretations have been proposed.

Results: Kinetics

**Charge recombination in the absence of $Q_B$ ($P^+Q_\lambda^-$ charge recombination; $k_p^\lambda$):**

As supported by my previous work presented in chapter 3, the His-tag does not significantly affect electron transfer kinetics in RCs. M265IN showed an ~2 fold increase in $k_p^\lambda$
when compared to His-WT (17.1 s\(^{-1}\) in M265IN vs. 9.1 s\(^{-1}\) in His-WT at pH 8.0) (Figure 17). These results are not significantly different from the \(k_{f,A}\) of M265IS and M265IT. For M265IQ, \(k_{f,A}\) was \(\sim\)30% slower (6.2 s\(^{-1}\) in M265IQ at pH 8.0) than His-WT at all pH values measured (Figure 17). For both M265IN and M265IQ, \(k_{f,A}\) was relatively pH independent from pH 4-8.5, but the charge recombination rates increased above pH 8.5. The pH significantly accelerates charge recombination above pH 9.5 in His-WT and the amide mutants.

**Biphasic nature of \(Q_A\) charge recombination:**

Previous work showed that the M265IS and M265IT \(k_{f,A}\) could be separated into a fast and slow component (Mattis and Wraight manuscript in preparation). The \(Q_A\) back reaction of M265IN and M265IQ could also be separated into two components. However, the amplitudes of the fast vs. slow components of M265IN and M265IQ varied far more over the pH range measured than those of M265IS and M265IT. The fast component for M265IN started out at 23% at pH 4.0 and continually increased until it plateaued around 75% at pH 7.5, staying relatively flat up to pH 11. At pH 7.0 the rates were 8.8 s\(^{-1}\), 31.6 s\(^{-1}\), and 18.4 s\(^{-1}\), for the fast, slow, and weighted averages respectively (Figure 18). The slow component was approximately 14 s\(^{-1}\) at low pH and gradually decreased to 6.4 s\(^{-1}\) at pH 8.0. Above pH 8.0 the slow component gradually increased in speed up to 15.5 s\(^{-1}\) at pH 11.0. The fast component also had a “bowl shaped” curve with a rate of 45.0 s\(^{-1}\) at pH 4.0 that decreased immediately with increasing pH and plateaued in the range of 26-32 s\(^{-1}\) \(k_{f,A}\) from pH 4.5 to 9.5 (Figure 18). Above pH 9.5 the fast component continued to increase with pH reaching 75.1 s\(^{-1}\) at pH 11.0.

The fast and slow component curves for M265IQ were inverted compared to M265IN. The fast component begins at 70% and gradually decreases to 20-30% by pH 6.0 (Figure 19). It
hovers in this range until pH 10.0 when it drops to around 10% for the rest of the pH values measured. In this case the slow component dominates most of the weighted average and this can be seen in Figure 19 with the slow component being almost identical to the weighted average from pH 6.0 and up. The slow component is quite slow for a Q\textsubscript{A} - back reaction at pH 4.0 (2.1 s\textsuperscript{-1}), but, immediately jumps up to a rate of 3 s\textsuperscript{-1} at pH 4.5 and stays relatively stable until pH 6.0 where it jumps again up to 4.7 s\textsuperscript{-1}. Above pH 6.0 the slow component is relatively unchanged until pH 8.5 when it begins to gradually increase reaching a rate of 8.6 s\textsuperscript{-1} at pH 11.0. The fast component is at 8 s\textsuperscript{-1} at pH 4.0 and increases until pH 6.5 where it stays relatively flat at a rate of approximately 12.5 s\textsuperscript{-1}. At pH 10.5 the fast component significantly increases in recombination rate to \approx 21 s\textsuperscript{-1}.

**Charge recombination in the presence of Q\textsubscript{B} (P^\textsuperscript{+}Q\textsubscript{A}Q\textsubscript{B}^{-} \rightarrow PQ\textsubscript{A}Q\textsubscript{B}^{-}; k_{p}^{B}):**

M265IN and M265IQ both showed a slower $k_{p}^{B}$ compared to His-WT. The $Q_{B}^{-}$ charge recombination for M265IN was 6-7 times slower than WT (0.15 s\textsuperscript{-1} for M265IN vs 0.95 s\textsuperscript{-1} for His-WT at pH 8.0), not significantly different from M265IT. $k_{p}^{B}$ of M265IQ was \approx 50% slower than His-WT (0.52 s\textsuperscript{-1} vs 0.95 s\textsuperscript{-1} at pH 8.0). Both mutants followed the same pH dependence as His-WT with pH dependent regions from pH 4-5.5 and pH 9.5-11.0, with rates increasing with increasing pH. pH independence was observed from pH 6.0-9.0 (Figure 20).

**Apparent 1\textsuperscript{st} electron transfer equilibrium constant between Q\textsubscript{A} and Q\textsubscript{B} (Q\textsubscript{A}Q\textsubscript{B}^{-} \rightarrow Q\textsubscript{A}Q\textsubscript{B}^{-}; k_{p}^{A})**:

The apparent equilibrium constant ($K_{AB}^{(1)}$) between $Q_{A}Q_{B}^{-} \rightarrow Q_{A}Q_{B}^{-}$ can be estimated by the ratio ($K_{AB}^{(1)} \approx k_{p}^{A}/k_{p}^{B}$ -1) of the rates of $k_{p}^{A}$ and $k_{p}^{B}$ [2]. M265IN had a 13-15 times greater apparent equilibrium constant than His-WT, but was not significantly different from M265IT.
(111 in M265IN vs. 133 in M265IT at pH 8.0) (Figure 21). M265IQ had an apparent equilibrium constant not significantly different from His-WT (10.7 in M265IQ vs. 8.7 in His-WT, at pH 8.0). $K_{AB}^{(1)}$ of M265IN followed the same trend in pH dependence as M265IT, decreasing from pH 4.0-5.5 and decreasing above pH 10.5. The apparent first electron transfer equilibrium for M265IN showed pH independence from pH 6.0-10.5. $K_{AB}^{(1)}$ of M265IQ followed a similar trend as His-WT, showing pH dependent regions from pH 4.0-5.5 and 10.0-11.0 (9.5-11.0 in His-WT), with the apparent equilibrium constant decreasing with increasing pH. $K_{AB}^{(1)}$ of M265IQ was pH independent from pH 6.0-9.5 (6.0-9.0 in His-WT).

**1st electron transfer** ($Q_A^{-}Q_B \rightarrow Q_AQ_B^-; k_{AB}^{(1)}$):

Both amide mutants showed 1st electron transfer kinetics that followed the same trend (Figure 22). The kinetics were pH independent from pH 4.0-8.5 and pH dependent from pH 9.0-11, where the 1st electron transfer became significantly slower with increasing pH. M265IN and M265IQ appeared to display approximately the same kinetics as one another, with 1st electron transfer rates almost 2 fold faster than His-WT below pH 9.0. At pH 9.0 and above, the 1st electron transfer kinetics for M265IN and M265IQ were not significantly different from His-WT.

**2nd electron transfer** ($Q_A^{-}Q_B^- \rightarrow Q_AQ_BH_2; k_{AB}^{(2)}$):

The 2nd electron transfer rates varied quite a bit between the RCs measured, but they all followed the same trend of decreasing rate with increasing pH (Figure 22). The $k_{AB}^{(2)}$ of M265IN was not significantly different from NT-WT kinetics. $k_{AB}^{(2)}$ of M265IQ was 4 fold slower than NT-WT at lower pH values, but became not significantly different at pH 9.5 and above.
Results: X-ray Crystal Structures

M265IN and M265IQ structures and significant $Q_A$ site changes:

The crystals for M265IN and M265IQ were tetragonal. Both crystals diffracted in the $P_{3121}$ space group, with unit cell dimensions of $a=b \approx 139.63 \pm 0.11 \ \text{Å}$ and $c \approx 184.53 \pm 0.55 \ \text{Å}$ (Table 4). The rms bond length deviation was at most 0.022 among the structures and the rms bond angle deviation was at most $2.38^\circ$. The number of Ramachandran violations varied from 24 to 33 in the structures. For purposes of comparison the 1DV3 structure was aligned with each of the mutants in VMD and the Ile-M265 has been left visible in the crystal structures shown in Figure 23 [3, 4]. The crystal structures with their respective changes compared to WT for $Q_A$ are reported below.

X-ray crystal structure of M265IN:

The structure of M265IN was solved to a resolution of 2.93 Å and refined to an R factor of 18.5% ($R_{\text{free}} = 22.6\%$) (Table 4). The C1 C=O quinone head group is H-bonded to the M260 peptide N with a length of 2.70 Å, which is not significantly different from the Xray-avg (2.78 ± 0.108 Å). However, the H-bond between the C4 C=O and $N_\delta$ of His-M219 was the longest of the four structures reported here at 3.06 Å, significantly different from the Xray-avg (2.82 ± 0.133 Å) (Figure 23). A weak H-bond may be present between the C4 C=O of the quinone head group and the O-H of Thr-M222 (3.36 vs. 3.69 ± 0.158 Å in Xray-avg). The orientation of the Asn side chain of M265IN follows a similar path as the $\gamma_1$ and $\delta$ carbons of Ile-M265 in 1DV3 (Figure 23). The Asn-M265 side chain has two heavy atom interactions at < 3.1 Å. These are either sterically repulsive, the amide carbonyl of the Asn side chain of M265 and the C4 C=O of
the quinone head group, at 3.02 Å, or attractive, a potential H-bond between amide C=O and N$_\delta$ of M219, at 2.80 Å (Figure 24).

**X-ray Crystal Structure of M265IQ:**

The Gln side chain of M265IQ is the largest of the M265 mutant side chains studied here and the most complex. The M265IQ structure was solved to a resolution of 2.74 Å and refined to a R factor of 18.9% ($R_{\text{free}}$ = 22.3%) (Table 4). The Gln side chain of M265 in the M265IQ mutant had two different conformations with approximately equal occupancy in the solved structure. Conformation A (Conf-A) has the Gln side chain following the same general path and direction as the WT Ile (γ1 and δ carbons in the 1DV3 structure) [3]. Conformation B (Conf-B) has the M265 Gln side chain rotated approximately 99° from Conf-A, pointing towards the quinone head group (Figure 25). In the crystal structure, Q-10 was also found to have an occupancy of approximately 50%. Incomplete occupancy of the $Q_A$ site was also apparent in flash spectroscopy experiments (data not shown). The side chain C=O of Conf-B is 2.17 Å from where the C4 C=O of the quinone head group would be and 2.42 Å from where the C3 methyl group of the quinone head group would be. These steric interactions likely lead to displacement of the quinone. The M265 side chain amide N of Conf-B has a H-bond to the C=O of Thr-M261 (2.82 Å). In Conf-A the amide N of the M265 Gln side chain is rotated away from the quinone head group such that it forms an internal H-bond with the backbone C=O (2.64 Å). The amide C=O of M265 in Conf-A points towards the quinone head group and is 3.01 Å from the C4 C=O of the quinone head group. The amide C=O also forms a potential H-bond with the N$_\delta$ of His-M219 (3.26 Å). The H-bond between $Q_A$ C1 C=O and the M260 backbone nitrogen is significantly shortened in the M265IQ structure to 2.41 Å ($X_{\text{ray-avg}} = 2.78 \pm 0.108$ Å), while
the H-bond between the $Q_A$ C4 C=O and the N$_\delta$ of His-M219 is significantly longer, at 3.04 Å (2.82 ± 0.133 Å in Xray-avg) (Figure 23).

**Discussion: Kinetics**

$P^*Q_A^-$ charge recombination kinetics:

There are two routes of charge recombination from $Q_A^-$: one is via direct tunneling to $P^*$ and the other is a thermally activated process through the bacteriopheophytin (I) (Scheme 1) [5]. The major route at 298 K in WT *Rb. sphaeroides* is direct tunneling [6]. However, if the redox potential of $Q_A$ is sufficiently low, either by substitution with a low potential quinone, e.g., anthraquinone (AQ), or with a mutation that sufficiently lowers the in situ $E_m$ of $Q_A^-$, e.g., M265IT and M265IS, then the indirect path via I becomes significant. If I assume that $k_{AP}$ is not significantly changed in the M265IN mutant when compared to WT, as Takahashi et al. (2001) assumed for M265IT and M265IS, then it is reasonable to conclude that the faster $k_{PA}^A$ (18.7 s$^{-1}$) of M265IN (vs WT at 9.5 s$^{-1}$) has a significant contribution from the $Q_A^-$ charge recombination via the indirect thermally activated route. This allows us to use $k_{PA}^A$ (UQ) WT in our calculation of the in situ $E_m$ of $Q_A$ for M265IN (see below).

M265IQ shows an ≈40% slower charge recombination from $Q_A^-$ (5.76 s$^{-1}$) compared to WT (9.5 s$^{-1}$). This is the opposite of the other three polar mutants. Due to the significantly larger size of Gln in M265IQ compared to Ile in WT, the Gln displaces $Q_A$ approximately 50% of the time (Figure 25). This likely occurs as the side chain flips between Conf-A and Conf-B. However, it is not clear how far the quinone is displaced from the WT $Q_A$ position, i.e., if it is displaced to a variety of sites or completely displaced. For simplicity I propose a scheme with only two quinone binding sites for $Q_A$, and will refer to these two sites as the proximal
(approximately normal position) and the distal site ($Q_\lambda^\pm$). Although, $Q_\lambda^\pm$ could be a combination of many different sites or even completely displaced.

With the assumption of a proximal and distal site for M265IQ I get Scheme 2. Successive flashes were shown to increase the amplitude of the absorbance for $P^+$ (data not shown). This indicates that there are at least two positions for $Q_\lambda$ and that one is not reducible by $I^-$. There are two possible explanations for this result. One is that $Q_\lambda$ is completely dissociated into the quinone-membrane pool for the not reducible state. The second is that there is a distal site that is not reducible, but not completely dissociated either. Due to the large driving force (almost 0.5 eV) from $I^-$, it is unlikely that any quinone bound near the $Q_\lambda$ site could not be reduced by $I^-$. Furthermore, detergent titration experiments show a decreased absorbance for $P^+$ with increasing detergent (Figure 26). This supports the complete dissociation of $Q_\lambda$ from the RC as the quinone spends more time in the detergent micelle as the micelle increases in size and number. Based on this result and the reasoning above I conclude that the inactive $Q_\lambda$ must be completely dissociated ($Q_{\lambda\text{dis}}$). This however, does not rule out the existence of distal $Q_\lambda$ site where the quinone may be reducible by $I^-$. 

After reduction of $Q_\lambda$ in either the proximal or distal site, in the absence of a quinone in $Q_B$, it is possible for $Q_\lambda^-$ to either move to the distal or proximal site, depending on its starting position, or to tunnel back to $P^+$. Based on the observed slow rate of $Q_\lambda^-$ charge recombination, I conclude that charge recombination via the thermal route from either the distal site ($k_{\lambda\text{III}}$) or proximal site ($k_{\lambda I}$) does not happen to a significant extent in this mutant. Therefore the proposed electron transfer scheme would look like Scheme 3.

From Scheme 3, there are two possible explanations for a slower $k_{\text{P}A}^\lambda$ in M265IQ compared to WT. First, the electron from $Q_{\lambda}^\delta$ can tunnel directly to $P^+$ ($k_{\lambda\text{IP}}$) with a slower rate
due to an increased distance between the quinone and $P^+$. In this case, if the equilibrium between $Q^+_A$ and $Q_A^- (K_{A/A})$ was slower than $k_{A/P}$, $k_{p/A}$ would be a combination of the tunneling rates from the distal and proximal sites and potentially biphasic. If the equilibrium $K_{A/A}$ was fast, then I would see an average of the charge recombination rates. The second possibility is that $k_{A/P}$ is slower than the charge recombination via $k_{A/A}$ and $k_{AP}$. In other words, charge recombination from the distal site is too slow to make a significant contribution to $k_{p/A}$ and all the recombination occurs via $k_{AP}$. In this situation $k_{p/A}$ would be a combination of $k_{AP}$ and $K_{A/A}$. Which scenario applies depends on the distance from $P^+$ of the distal site(s), the electrostatic environment of the distal site(s), and the $E_m$ of the quinone bound in the distal site(s). If the electron on $Q^+_A$ cannot tunnel directly back, then the only route of charge recombination is via the proximal quinone binding position and the slower rate I observe is caused by the rate of movement of the semiquinone from the distal to the proximal site of $Q_A$.

**In situ $E_m$ of $Q_A$ in Mutant RCs:**

$k_{p/A}$ is a combination of two routes ($k_{AP}$ and indirect via $I$) and the rate expression is:

$$k_{p/A} = k_{ip} \exp[\Delta G_{IA}/RT] + k_{AP} = k_{ip}(1 + K_{IA}) + K_{AP}K_{IA}/(1 + K_{IA})$$

where $\Delta G_{IA} = -RT \ln K_{IA}$ is the free energy gap between $IQ_A$ and $IQ_A^-$ [5], equivalent to the difference in $E_m$ between $I/I$ and $Q_A^-/Q_A$. If the $E_m$ of $I/I$ is assumed to be unchanged by the M265 mutations, $\Delta G_{IA}$ can be used to determine if there is a change in the in situ $E_m$ of $Q_A^-/Q_A$ of the M265 mutants, compared to WT. The rate of $P^+I^-$ charge recombination, $k_{ip} = 2 \times 10^7$ s$^{-1}$, is independent of $Q_A$ mutations or quinone identity [5]. For WT RCs, $\Delta G_{IA} = -470$ meV. With the assumption above that $k_{p/A}$ (UQ) is faster for the M65IS, M265IT, and M265IN mutants due to the indirect route contribution to $k_{p/A}$, I can now calculate the difference in $\Delta G_{IA}$ between the
mutant and WT RCs ($\delta G_{I\alpha}$). Using the WT and mutant difference of $k_p^A$, $\Delta k_p = k_p^A$ (mut) – $k_p^A$ (WT), I obtain: $\delta G_{I\alpha} = RT \ln (\Delta k_p/k_p^A) - \Delta G_{I\alpha}$. With $\Delta G_{I\alpha} = -470$ meV, I obtain $\delta G_{I\alpha}$ of 89 meV for M265IN. Assuming that the entire change in $E_m$ is due to changes at $Q_A$, rather than I, this translates to a drop of in situ $E_m$ of $Q_A$ of 89 meV. A comparable result can be calculated using $\Delta E_m = -56.6 \log (k_{PA}^{-7}) – 53.1$ mV [7]. These values are very close to those obtained for the non-his-tagged versions of M265IS and M265IT [1]. Note that neither of these methods can be used to estimate the in situ $E_m$ of $Q_A$ for M265IQ because $k_p^A$ is so much slower.

The addition of a single polar group at M265 in place of the non-polar Ile has a very large effect to the $E_m$ of $Q_A$. Based on the lowered $E_m$ of $Q_A$ and the weaker quinone binding at $Q_A$, I conclude that the semiquinone is more destabilized in the $Q_A$ site than the oxidized form of the quinone for the M265IS, M265IT, and M265IN mutants. Some of the possible causes of the difference are a change in the polarity or dielectric of the $Q_A$ site due to the addition of the polar side chain and possible structural changes or electron distribution in $Q_A$ due to new H-bonds formed near $Q_A$. To provide further insight, I look to the X-ray crystal structures discussed below.

**$P^*Q_AQ_B^-$ Charge Recombination Kinetics ($k_p^B$):**

$Q_B^-$ charge recombination can be approached using a similar analysis as for $Q_A^-$ charge recombination. Charge recombination from $Q_B^-$ can occur via the direct tunneling route to $P^*$ or through a thermal repopulation of $Q_A^-$ and then recombination from $Q_A^-$, as discussed above (Scheme 4). The observed rate of charge recombination from $Q_B^-$ ($k_p^B$) is expressed as:

$$k_p^B = k_{IP} \exp[-\Delta G_{IB}/RT] + k_p^A \exp[-\Delta G_{AB}^{(1)}/RT] + k_{BP}$$

$$\approx k_p^A \exp[\Delta G_{AB}^{(1)}/RT] + k_{BP}$$
\[ k_P^A / (1 + K_{AB}^{(1)}) + k_{BP} K_{AB}^{(1)}/(1+K_{AB}^{(1)}) \] (2)

The contribution from \( k_{BP} \) is negligible (<0.02 s\(^{-1}\)), since \( \Delta G_{IB} \leq -540 \text{ meV} \) [2], and the direct tunneling route from \( Q_B^- \) (\( k_{BP} \)) is very slow (0.1-0.12 s\(^{-1}\)) in WT RCs at pH 7.0 [8]. Assuming the \( E_m \) difference between \( Q_A^-/Q_A \) and \( Q_B^-/Q_B \) is not too large and therefore \( K_{AB}^{(1)} \) is not too large (\( K_{AB}^{(1)} \approx 15 \) in WT RCs), \( k_{BP} \) can be ignored as it is approximately 100 times smaller than \( k_P^A \) and therefore not a significant route of charge recombination. With this assumption, I can estimate \( K_{AB}^{(1)} \) by rearranging the equation above:

\[ K_{AB}^{(1)} \approx (k_P^A / k_P^B) - 1 \] (3)

However, the M265IS, M265IT, and M265IN mutants all have a \( k_P^B \) that are significantly slower than WT. Thus \( k_{BP} \) does play a significant role in \( Q_B^- \) back reaction kinetics for these mutants and cannot be ignored. Therefore equation 3 gives the lower limit of the equilibrium potential, which can be used to solve for the minimum \( E_m \) difference between \( Q_A^-/Q_A \) and \( Q_B^-/Q_B \) for these three mutants [1]. Solving for \( \Delta G_{AB}^{(1)} \) and ignoring \( k_{BP} \), yields values of -114, -129, and -126 meV for M265IS, M265IT, and M265IN respectively. These correspond to a drop in \( E_m \) of \( Q_A \) of 56, 72, and 69 meV, respectively.

M265IQ has a slower \( k_P^B \) (0.46 s\(^{-1}\)) than WT (0.91 s\(^{-1}\)). Using equation 3 above \( K_{AB}^{(1)} \) for M265IQ is 11.5. This is not very different from WT (9.1) and therefore should be acceptable to determine the \( E_m \) difference between \( Q_A^-/Q_A \) and \( Q_B^-/Q_B \) without concern for the direct route of recombination from \( Q_B^- \). Solving for \( \Delta G_{AB}^{(1)} \) and ignoring \( k_{BP} \) I get -65.9 meV which is 8.5 meV lower than His-WT. This is not significantly different and the change is likely limited to changes to \( Q_A \). Based on the \( K_{AB}^{(1)} \) of M265IQ the slower \( k_P^B \) is caused by the slower \( k_P^A \) discussed above.
The first electron transfer ($Q_A^-Q_B^+\rightarrow Q_A^+Q_B^-$):

The first electron transfer kinetics are known to be independent of the $E_{m}$ difference between $Q_A^-/Q_A$ and $Q_B^-/Q_B$ [9]. Rather, electron transfer to $Q_B$ has been proposed to be at least partially controlled by the electrostatic potential generated in the $Q_B$ site by a cluster of acidic residues including Glu-L212 [2, 10-16]. The cluster of acidic residues has a pK of approximately 9.0. The fraction of ionizable atoms in the protonated state determines the rate of 1st electron transfer and when above the apparent pK, the rate decreases in proportion to the pH [6, 17]. There have been many structure-based electrostatic calculations performed that support the $Q_B$ acid cluster idea [18-21].

The acid cluster that controls the rate of 1st electron transfer between $Q_A$ and $Q_B$ is in the local region of $Q_B$ and the previously published M265IS and M265IT pH dependence trends were surprising because the mutations caused such a significant change to the pK. Previously published results showed a lower pH dependent region for NT-M265IT and NT-M265IS from pH 7.0 to 9.5, with a second pH independent region (pH 10.0-11.0) [1]. However, the 1st electron transfers of the His-tagged M265 mutants showed the same pH dependence trends (4.0-8.5 pH independent and 9.0-11.0 pH dependent) as WT and His-WT (Figure 22). The only differences between M265IT and M265IS in Takahashi et al. (2001) and this work are the 6xHis-tags. After carefully repeating the experiments with NT-M265IT I did not see the pH dependence previously published (data not shown). A pH dependence trend similar to what was observed by Takahashi et al. became apparent when a two-component analysis was taken and only the slow component was used. Based on this, the different trends are based on how the data was analyzed, rather than the addition of the 6xHis-tag. I believe the data presented here is
correct, which is consistent with the mutations being in the QA site and therefore not affecting the 1st electron transfer kinetics.

The M265IQ mutant showed 1st electron transfer kinetics that were not significantly different from the other mutants or WT. This is not surprising considering that 1st electron transfer is generally driving force independent.

**Second electron transfer (QA·QB → QAQBH₂)**

The second electron transfer (k_{AB}^{(2)}) is tightly coupled with proton delivery to QB. There are two proposed sequences: electron transfer to QB first, followed by proton transfer (ET/PT), or proton transfer first, followed by electron transfer (PT/ET) (Scheme 5; top right vs bottom left). Graige et al observed that by varying quinone analogs in QA, the rate of second electron transfer is dependent on the driving force of the reaction. Graige and coworkers were able to determine that the mechanism of second electron transfer proceeds via the PT/ET route [22, 23].

Second electron transfer kinetics are 2-4 times faster for M265IN than M265IQ or His-WT consistent with a larger driving force arising from the lowered E_m of QA for the M265IN mutant, but not the M265IQ mutant. k_{AB}^{(2)} for M265IQ was not significantly different from His-WT, consistent with the small change of 8 meV of E_m for QA reported above.

**Discussion: X-ray Crystal Structures**

**M265IN X-ray Crystal Structure:**

The kinetics of M265IN are not significantly different from those of the other hydroxyl mutants M265IS and M265IT. Asn has a larger side chain and a different polar group than Thr and Ser (amide vs hydroxyl). However, since the kinetics of these mutants are very similar, it is
reasonable to assume that there is a similar mechanism for all three polar mutants in regards to the kinetic differences seen compared to WT. Even with the significantly larger side chain of Asn, the binding affinity for $Q_A$ did not seem to vary from M265IS or M265IT. As in M265IS and M265IT, it is likely that the placement of the polar group of Asn in M265IN near the quinone in the $Q_A$ site exerts a change in the electrostatic environment that alters the midpoint potential of $Q_A/Q_A^-$. However, the structure of M265IN suggests the situation may be a bit more complicated, as discussed below.

The methoxy dihedral angles from the X-ray structure of M265IN are 135.45° and -76.26°, 2 and 3 methoxy respectively. These are not significantly different from the WT-Avg angles reported by Wraight and Gunner (139 ± 25° and -77 ± 8°)[24]. Therefore these dihedral angles are likely not significant contributors to the change in $E_m$ of $Q_A/Q_A^-$. 

The Asn side chain of M265IN mostly overlaps with the Ile of 1DV3 when the structures are aligned (Figure 24). The $\beta$ carbons of the two structures are almost in an identical position. However, the Asn $\beta$-$\gamma$ carbon bond is rotated by 17.4° away from the quinone head group (towards the back bone C=O of M265), possibly to avoid displacing $Q_A$. The rotation of the $\beta$-$\gamma$ carbon bond places the $\gamma$ carbon of the Asn-M265 side chain approximately halfway between the position of the $\gamma_1$ carbon and the $\delta$ carbon of Ile-M265. With the amide oriented as shown in Figure 24, the C=O group of the Asn side chain is pointed towards the C4 carbonyl of the quinone head group and the $N_\delta$ of M219 histidine. This positions the oxygen of the side chain C=O of Asn-M265 only 3.02 Å away from the C4 carbonyl of the quinone head group. These two groups would repel one another, which may cause the small 4.0° tilt and rotation seen in the $Q_A$ head group of the M265IN X-ray structure. In this orientation, the Asn-M265 side chain carbonyl forms a H-bond with the $N_\delta$ His-M219, which is shorter in length (2.80 Å) than the
The H-bond between the C4 carbonyl of the quinone head group and the N$_\delta$ His-M219 (3.06 Å). The H-bond between the quinone C4 C=O and N$_\delta$ His-M219 is longer for two reasons. First, the steric clash with the side chain C=O of Asn-M265 would push the quinone head group away from the His-M219, increasing the H-bond length. Second the H-bond between the Asn-M265 side chain C=O and N$_\delta$ His-M219 is shorter and therefore stronger than the His-M219/C4 quinone H-bond. Bifurcation of a H-bond reduces the strength of each H-bond by up to one half. Addition of a second H-bond with N$_\delta$ His-M219, would cut the strength of the quinone C4 C=O H-bond accordingly. This reduces the binding strength of the quinone to the QA site, while the steric clash of the two carbonyls (C4 of the quinone head group and side chain Asn-M265) pushes the quinone farther away from His-M219.

The Asn-M265 amide nitrogen is 4.1 Å to the nearest heavy atom of the quinone head group. Therefore the Asn-M265 amide N does not interact much beyond van der Waals interactions with the QA head group. However, the N is 3.04 Å from the backbone carbonyl of M265, forming a possible H-bond. The angles of this potential H-bond are 52.45°. The side chain amide N is within van der Waals distance of C5 and C6 of the quinone head group, the methyl of Ala-M260, and the β carbon of Trp-M268. The van der Waals interactions add stability to the QA binding pocket, which M265IT and M265IS have lost. However, this may be overcome by some of the steric clashes discussed above.

The C4 carbonyl of the quinone head group and the hydroxyl of Thr M222 are also within H-bonding distance at 3.36 Å (Xray-Avg: 3.69 ± 0.16) with an angle of 123.7°. If this is a H-bond it will likely be weak, but may also contribute to the increased length of the C4 carbonyl-His-M219 H-bond. It may also increase the binding affinity of the quinone to make up for the weakened H-bond between C4 C=O and His-M219 N$_\delta$. The H-bond between C1 carbonyl of the
quinone head group and the backbone N of Ala-M260 is not significantly different from Xray-Avg (2.70 vs. 2.78 ± 0.11).

As it is not possible from X-ray diffraction data to determine the correct rotamer of the amide group on Asn, it is possible that the N and carbonyl groups of the side chain of Asn-M265 could be switched. The side chain N of Asn-M265 would then interact with N\(_{\delta}\) His-M219. It is possible for a H-bond to form between a N\(_{\delta}\) of a His and the N, but it is more likely that this interaction would have steric clashes and be unfavorable. If this rotamer were correct, there would now be a H-bond between the side chain N of M265 and the C4 C=O of the quinone head group. However, if this were the case, the quinone head group would not be closer to Thr-M222 as the x-ray model shows. Therefore I believe the orientation presented above, and shown in Figures 23 and 24, is the correct rotamer.

In addition to the structural and quinone binding changes discussed above, there are also the electrostatic effects of adding the amide group near Q\(_A\). The amide group of M265IN is a stronger polar group than the hydroxyl of M265IS or M265IT. Therefore I would expect there to be a greater effect on the dielectric of the Q\(_A\) site. The interplay of the conformation of the methoxy groups, the change in polarity of the Q\(_A\) site, and the changes to binding affinity for quinones in the Q\(_A\) site control the redox potential of Q\(_A\). For M265IN, it seems that only the later two play a part. To parse out individual contributions, electrostatic calculations using computational methods are needed.

**M265IQ Crystal Structure:**

M265IQ has two conformations, one with the quinone present in Q\(_A\) and one where the quinone is absent. The displacement of the quinone in the Conf-B is the cause for the difference
in back reaction kinetics observed. The major reason for the displacement is the size of the Gln side chain. Gln has an additional methylene group compared to Asn. This increase in size partially displaces the quinone from the $Q_{\alpha}$ site and/or makes it more difficult for the quinone to stay bound. This result is apparent in both the crystal structure and in the sequential flash experiments (data not shown) discussed above.

The side chain of Gln-M265 in Conf-A of M265IQ overlaps strongly with Ile in 1DV3, although the C$\gamma$2 of Ile-M265 does not overlap with any atoms from the Gln-M265 crystal structure. However, the $\beta$, $\gamma1$, and $\delta$ carbons of Ile-M265 occupy almost the same position as the $\beta$, $\gamma$, and $\delta$ carbons of Gln-M265. The Gln-M265 $\gamma$C-$\delta$C bond is deviated by 12.5° from Ile-M265 in 1DV3. These similar heavy atom positions in Conf-A largely preserve the van der Waals contacts that Ile-M265 has with $Q_{\alpha}$. However, even in Conf-A there is an unfavorable steric interaction present. The amide N of the Gln-M265 side chain is 2.62 Å from the C5 methyl group of the $Q_{\alpha}$ head group. The side chain N of Gln-M265 is also 2.99 Å from C5, 3.23 Å from C4, and 3.01 Å from the C4 C=O. This last interaction is a potential H-bond. The other three interactions with the N of the Gln-M265 side chain sterically clash and are part of the reason the quinone is displaced. The carbonyl of the M265 amide is also 2.64 Å from the backbone C=O of M265. This interaction is unfavorable and would also destabilize Conf. A.

The methoxy dihedral angles from the X-ray structure of M265IQ are 150.76° and -99.57°, 2 and 3 methoxy respectively. The 3 methoxy dihedral is therefore significantly different from the WT-Avg angles reported by Wraight and Gunner (139 ± 25° and -77 ± 8°) (Wraight and Gunner 2009). This could indicate that the small change we see in $E_m$ of $Q_{\alpha}$/2$Q_{\alpha}$ could be due to the 3 methoxy orientation.
Since the orientation of amides is not crystallographically resolvable, I also much entertain the opposite orientation. This would now put the side chain carbonyl of Gln-M265 within 3.01 Å from the C4 C=O. This interaction is at least partially responsible for the initial displacement of Q$_A$ that allows for M265IQ to move to Conf. B. Therefore it is not clear which orientation for Conf. A is correct. It is possible that both are equally possible.

**Conclusions:**

The X-ray structures give a snapshot of the ground state of the M265 amide mutants, M265IN and M265IQ. The M265IS and M265IT structures show that small structural changes lead to significant changes to the redox potential of Q$_A$. For the hydroxyl mutants, the most significant contribution to the change in redox potential for Q$_A$ is likely the change in quinone binding and change in polarity of the Q$_A$ site. The story with the amide mutants, M265IN and M265IQ, is more complex. Due to both Asn and Gln being larger and more polar than the Ser and Thr mutants I see many more changes. For M265IN there are significant changes in the quinone position, steric interactions with Q$_A$, and probably changes in the electrostatic potential, though the latter needs to be calculated to determine its actual contribution. M265IQ is especially complex as it also has the 3 methoxy group significantly rotated, with an increased size of the side chain that can take on 2 conformations with one that displaces the quinone from Q$_A$. M265IQ is strange because it does not appear that the redox potential is altered by the mutation. Perhaps there are evolutionary pressures that are energetic or structural in nature that keep the RC functioning.

Ultimately the X-ray structures are useful when examining the quinone in their ground state, but not very useful when trying to compare the charge separated or semiquinone states.
Other techniques specifically suited to studying charged states, such as electron pulse resonance are needed to assess the semiquinone effects for these mutants. These additional studies, as well as electrostatic potential calculations using the crystal structures are the next steps to provide further evidence that the dielectric environment is the largest contributor to determining $E_m$.

References:


8. Labahn, A., et al., *Direct Charge Recombination from D(+)Q(a)Q(B)(-) to Dq(a)Q(B) in Bacterial Reaction Centers from Rhodobacter-Sphaeroides Containing Low Potential Quinone in the Q(a) Site*. Chemical Physics, 1995. 197(3): p. 355-366.


Figure 17: pH dependence of $P^+Q_A^-$ charge recombination monitored by the disappearance of $P^+$ at 430nm, in the presence of 50μM terbutryn to block $Q_B^-$ formation. NT-WT data are here for comparison from Takahashi et al 2001.
**Figure 18:** Biphasicity of $k_p^A$ for M265IN. The top graph shows the slow vs fast component percentages at each pH value measured. The bottom graph shows the rate of the fast, slow, and weighted average components over the entire pH range.
Figure 19: Biphasicity of $k_p^A$ for M265IQ. The top graph shows the slow vs fast component percentages at each pH value measured. The bottom graph shows the rate of the fast, slow, and weighted average components over the entire pH range.
Figure 20: pH dependence of $P^+Q_AQ_B^-$ charge recombination monitored by the disappearance of $P^+$ at 430nm, in the presence of 20μM Q-10 to increase the likelihood of $Q_B$ being occupied by Q-10. NT-WT data are here for comparison from Takahashi et al 2001.
Figure 21: pH dependence of the apparent equilibrium constant between $Q_A^{-}Q_B$ and $Q_AQ_B^{-}$. NT-WT data is here for comparison from Takahashi et al 2001.
Figure 22: pH dependence of 1st and 2nd electron transfers in M265 mutants. NT-WT and NT-M265IT data are here for comparison from Takahashi et al. 2001.
### Table 4

This table lists the summary of results from the analysis of the X-ray crystal structures of the four polar M265 mutants.

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#### Crystal Data

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<td>2.93</td>
<td>2.74</td>
</tr>
<tr>
<td>Unique Reflections</td>
<td>53634</td>
<td>42918</td>
<td>44997</td>
<td>54952</td>
</tr>
<tr>
<td>Completeness (highest shell) (%)</td>
<td>99.5 (100.0)</td>
<td>99.2 (99.9)</td>
<td>99.6 (99.97)</td>
<td>99.4 (100.0)</td>
</tr>
<tr>
<td>Average I/σ (last shell)</td>
<td>11.77 (2.14)</td>
<td>15.79 (2.22)</td>
<td>13.37 (2.89)</td>
<td>12.24 (2.70)</td>
</tr>
<tr>
<td>R merge (last shell)</td>
<td>0.105 (0.755)</td>
<td>0.094 (0.762)</td>
<td>0.125 (0.793)</td>
<td>0.123 (0.758)</td>
</tr>
</tbody>
</table>

#### Refinement

| R Factor | 0.191 | 0.174 | 0.185 | 0.189 |
| R Free | 0.227 | 0.194 | 0.226 | 0.223 |
| From Wilson Plot (B-value) | 59.93 | 62.36 | 55.99 | 55.11 |
| Average B value (Å²) | 50.00 | 53.84 | 48.49 | 44.85 |
| rms bond Length deviation (Å) | 0.024 | 0.035 | 0.021 | 0.022 |
| rms bond angle deviation (°) | 2.580 | 3.196 | 2.364 | 2.380 |
| Error in coords by Luzzati plot (Å) | 0.320 | 0.327 | 0.331 | 0.312 |
| Ramachandran Violations | 31 | 42 | 33 | 24 |
Figure 23: The QA site of the X-ray crystal structures of all four polar M265 mutants are shown in this VMD representation. Heavy atom interactions of interest are indicated with arrows between them and the distance between the two heavy atoms is given in Ångstroms. Numbers in parentheses below a distance are the Xray-avg distance. Distances are either black for not significantly different or red for significantly different from Xray-avg. Distances without values in parentheses below did not have corresponding values from Xray-avg. These are given in red to highlight the short distance, which may either be H-bonds or steric repulsions. The Ile-M265 amino acid from the 1DV3 structure is present in gray for comparison. The structures were aligned using SEQ in VMD [4].
Figure 24: The QA site of M265IN with a comparison to the 1DV3 structure. Heavy atom interactions of interest are indicated with arrows between them and the distance between the two heavy atoms is given in Ångstroms. Numbers in parentheses below a distance are the Xray-avg distance. Distances are either black for not significantly different or red for significantly different from Xray-avg. Distances without values in parentheses below did not have corresponding values from Xray-avg. These are given in red to highlight the short distance, which may either be H-bonds or steric repulsions. Parts of the 1DV3 structure are present in gray for comparison. The structures were aligned using SEQ in VMD [4].
Figure 25: Conformation A (left) & Conformation B (right) of the M265IQ X-ray crystal structure. Heavy atom interactions of interest are indicated with arrows between them and the distance between the two heavy atoms is given in Ångstroms. Numbers in parentheses below a distance are the Xray-avg distance. Distances are either black for not significantly different or red for significantly different from Xray-avg. Distances without values in parentheses below did not have corresponding values from Xray-avg. These are given in red to highlight the short distance, which may either be H-bonds or steric repulsions. The Ile-M265 amino acid from the 1DV3 structure is present in gray for comparison. The structures were aligned using SEQ in VMD [4].
Scheme 1: $P^\cdot Q_A^{-}$ back reaction. Charge separation/recombination scheme for the RC in the absence of $Q_b$. $K_{IA}$ is the equilibrium constant between the $P^\cdot I^+ Q_A$ and $P^\cdot I^+ Q_A^{-}$ states. $k_{AP}$ is the direct tunneling rate constant and a combination of $K_{IA}$ ($L_{IA}$) and $k_{IP}$ (rate constant of charge recombination from $P^\cdot I^+ Q_A$) gives the thermally activated route, $k_{AIP}$ (see Figure 1).
Scheme 2: Electron transfer scheme for M265IQ for 1Q RCs. $Q^\dagger_A$ is the distal position(s) that the quinone may occupy and is taken as all other possible sites other than the proximal site. $k_{A^\dagger P}$ is the rate of direct tunneling charge recombination from $P^\dagger IQ^\dagger_A$ to $PIQ_A$. $L_{IA}$ is equivalent to $K_{IA}$. 
Figure 26: M265IQ titration with Triton X-100. The decrease in the initial absorbance change at 430 nm indicates that the quinone from QA is dissociating into the quinone-micelle pool. 50 μM terbutryn was present to block QB⁻ formation.
Scheme 3: M265IQ 1Q RC electron transfer and charge recombination, omitting the thermal recombination routes from $Q_A^-$ and $Q_A^{\pm}$, which are not needed to explain the observed kinetics.
Scheme 4: Charge recombination in the presence of Q_B. \( L_{AB}^{(1)} \) is equivalent to \( K_{AB}^{(1)} \).
Scheme 5: Two proposed paths for the 2\textsuperscript{nd} electron transfer are shown. Based on the work of Graige and coworkers, PT/ET is the dominant route for the 2\textsuperscript{nd} electron transfer.
Appendix A:
Additional Materials and Methods Details

Introduction:

Although *E. coli* are the most commonly used bacteria for studying prokaryotic proteins or systems, they lack all the biosynthesis pathways for the various cofactors and are therefore not yet suitable for expressing the bacterial photosynthetic reaction center (RC). For this reason I use the native species of bacteria that express the RC. Primarily these methods will focus on the purple non-sulfur bacteria *Rhodobacter sphaeroides* (*Rb. sphaeroides*).

This appendix will discuss materials and methods for complete details of mutagenesis, growth of wild type (WT) and mutant strains, purification of non-His-tagged RCs, and flash spectroscopy kinetic studies. Details of new experiments performed regarding purification of His-tagged RCs, crystallization of RCs, and X-ray crystallography data collection and analysis were discussed in chapter two of this dissertation.

Mutagenesis:

A construct previously used by Eiji Takahashi was used as the platform for the site directed mutagenesis [1, 2]. This construct consists of the 2686 basepair (bp) high copy plasmid pUC19 (New England Biolabs), which is very convenient for oligonucleotide site-directed mutagenesis. The construct contains an insert of part of the puf operon that includes the puf L and puf M genes (Figure 27) [3]. An EcoR I cleavage sites flank each side of the insert.

Site directed mutagenesis was performed using thermal cycling with the following mixture:
5 μL 10x polymerase buffer
1 μL dsDNA template (10-50ng/μL)
1 μL primer #1 (125 ng/μL)
1 μL primer #2 (125 ng/μL)
2 μL 12.5 mM dNTP mix (250 μM final)
1.5 μL DMSO³
37.5 μL H₂O
1 μL Pfu Ultra (Turbo) DNA polymerase (2.5 U/μL)

The dsDNA template was the pUC19 plasmid with the puf operon insert. Primers #1 & #2 were the forward and reverse primers, respectively, at the location of the intended mutation. They were designed with 8-10 nucleotides flanking around the three nucleotides that coded for the amino acid mutation of interest. The dNTPs (New England Biolabs: catalog # N0447S), buffer, and DNA polymerase were initially purchased seperately, but eventually a kit was used for site directed mutagenesis (New England Biolabs: catalog # E0553S). Early site directed mutagenesis of M265IT and M265IS was performed with PfuTurbo HS DNA polymerase (Agilent: catalog # 600410). All other site directed mutagenesis was performed with PfuUltra II Fusion HS DNA polymerase (Agilent: catalog # 600850) that has significantly higher fidelity than PfuTurbo.

Attempts were made to perform the mutation directly to the pRK415 plasmid with the insert (see below), but these were unsuccessful.

Desalted primers were from Integrated DNA Technologies (IDT). Twenty five nmoles of each primer were ordered. Water was added to the solid primers and the concentration was

³ Although certain protocols state that DMSO is optional, I found that the site directed mutagenesis would not occur without it.
checked and adjusted using their respective extinction coefficients, as reported by IDT. The primers were stored at -20 °C in stocks of 125 ng/μL.

**Primers:**

Note that capital letters represent the changed or added nucleotides.

**6x His-Tag Primer**

Primer Forward: 5’ gaa ctg aac CAC CAC CAC CAC CAC CAC tga gga tcg 3’

Primer Reverse: 5’ cga tcc tca GTG GTG GTG GTG GTG GTG gtt cag ttc 3’

**M265IS:**

Forward: 5’ cg atg gaa ggc aGe cac cgc tgg gc3’

Reverse: 5’ gc cca gcg gtg gCt gcc ttc cat cg3’

**M265IT:**

Forward: 5’ cg atg gaa ggc aCc cac cgc tgg gc3’

Reverse: 5’ gc cca gcg gtg gGt gcc ttc cat cg3’

**M265IN:**

Forward: 5’ cg atg gaa ggc aAc cac cgc tgg gc3’

Reverse: 5’ gc cca gcg gtg gTt gcc ttc cat cg3’

**M265IQ:**

Forward: 5’ cg atg gaa ggc CAG cac cgc tgg gc3’

Reverse: 5’ gc cca gcg gtg CTG gcc ttc cat cg3’
All the reagents were mixed on ice. The Mini Cycler (MJ Research) was used for thermal cycling with the following protocol:

<table>
<thead>
<tr>
<th>Step</th>
<th>Cycles</th>
<th>Temp</th>
<th>Time</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>94 °C</td>
<td>1 min</td>
<td>Melt dsDNA Template</td>
</tr>
<tr>
<td>2</td>
<td>12-18</td>
<td>94 °C</td>
<td>1 min</td>
<td>Melt dsDNA Template</td>
</tr>
<tr>
<td></td>
<td></td>
<td>52 °C</td>
<td>1 min</td>
<td>Hybridization</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68 °C</td>
<td>2 min/kb</td>
<td>Elongation</td>
</tr>
<tr>
<td>3</td>
<td>infinite</td>
<td>4 °C</td>
<td></td>
<td>Hold</td>
</tr>
</tbody>
</table>

Following completion of the thermal cycle, 1 μL of the restriction enzyme Dpn I (10 U/μL) was added to each reaction and incubated at 37 °C for 1-2 hours. This enzyme digests methylated DNA, which is only present on the original DNA template added to the site directed mutagenesis reaction. Since the new and hopefully mutated pUC19 plasmids have been made synthetically, they are unmethylated and therefore not broken down by Dpn I.

The DNA from the site directed mutagenesis was then transformed into *E. coli* XL1-Blue competent cells. Competent cells were made as follows:

**Rubidium Chloride competent cells:**

<table>
<thead>
<tr>
<th>Solution 1:</th>
<th>Solution 2:</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 mL solution in water</td>
<td>100 mL solution in water</td>
</tr>
<tr>
<td>KOAc: 0.588 g</td>
<td>MOPS: 0.21 g</td>
</tr>
<tr>
<td>RuCl₂: 2.42 g</td>
<td>RuCl₂: 0.121 g</td>
</tr>
<tr>
<td>CaCl₂: 2.0 g</td>
<td>CaCl₂: 1.1 g</td>
</tr>
</tbody>
</table>
MnCl₂: 2.0 g
Glycerol: 15 mL

Glycerol: 30 mL
Adjust to pH 6.5 with dilute NaOH

Adjust to pH 5.8 with dilute acetic acid
Filter sterilize and store at 4 °C

Filter sterilize and store at 4°C

1)  Innoculate 50 mL of Luria broth (LB) with 0.5 mL of cells and grow to $A_{550} = 0.48$

2)  Place on ice for 15 minutes

3)  Pellet cells in sterile tube @ 3500 rpm for 5 minutes

4)  Pour off supernatant and resuspend in 20 mL of solution #1

5)  Place on ice for 15 minutes

6)  Pellet cells in sterile tube @ 3500 rpm for 5 minutes

7)  Pour off supernatant and resuspend in 2 mL of solution #2

8)  Aliquot cells in increments of 100 μL and store at -80 °C

Transformation Protocols (Heat shock method):

1)  Thaw competent cells on ice.

2)  Aliquot 50 μL of competent cells into pre-chilled tubes on ice

3)  Add 7 μL of DNA to be transformed to 50 μL of competent cells.

4)  Let tube site on ice for 20-30 minutes.

5)  Heat shock competent cells by placing them at 42 °C for 50 seconds

6)  Return heat shocked competent cells to ice for 2 minutes

7)  Add 1 mL of Luria broth to heat shocked competent cells and plate cells
XL1-Blue cells have tetracycline resistance (Tc') and pUC19 carries an ampicillin resistance gene. So the transformed cells are plated onto LB plates with 15 μg/mL of tetracycline (Tc) and 200 μg/mL of ampicillin. The plates are then placed at 37 °C.

Luria broth medium and plates protocols:

For 1 L of broth:

10 g NaCl

5 g yeast extract

10 g tryptone

Add all of the above to 1 L of water

Adjust pH to 7.0 with NaOH and autoclave for 30 minutes.

After autoclaving, let cool to below 37 °C before use.

For Plates:

Follow broth recipe, but add 20 g of agar per liter and autoclave 1 L of media in a 2 L flask with a magnetic spinner for 30 minutes.

After autoclaving, incubate the flask in a 60 °C water bath so that the flask will cool, but the agar will not set. Forty-five minutes should be enough time for the flask to reach 60 °C.

If adding antibiotics, X-gal, or IPTG, follow the step below. Otherwise skip to pouring plates.

Adding antibiotics, X-gal, or IPTG:

Put the flask on a stir plate and stir slowly as not to cause bubbles. Add additives of choice using sterile technique and let stir for 1 minute after last addition. It is important that this is done once the flask has cooled to 60 °C, as the additive could be denatured by heat. This is specifically important for certain antibiotics.

Pouring plates:
Pour plates using sterile conditions and under a flame. 500 mL of LB agar will yield 12-14 medium sized plates (100 x 15 mm). Pour plates about 2/3 full. Plates that are too thin risk drying out and cracking, making them useless. These plates can be identified by not having a completely covered surface after being stored at 4 °C or large cracks in the agar. Discard any such plates if they are found. Once the plates set, they should be stored at 4 °C in the dark, upside down. They are usually good for at least 6 weeks. Be sure to check plates for cracking or contamination before use. Allow plates to warm up to room temperature (30 minutes on the bench or 20 minutes in the 37 °C incubator) when moving from 4 °C for use.

Two methods of plating cells were initially used. First, cells were plated using the glass pipet spreader method. In this method a glass pipet is melted to a right angle and used to spread the cells on the plate after being flamed. The problem is that there are often risks of cross contamination if the pipet is not flamed long enough, or damaging the cells with heat if there is not enough waiting time following the flaming of the glass spreader. An alternative method is the glass bead method in which 3-5 glass beads (autoclaved in a small bottle) are dropped onto the plate and the plate is shaken for 60 seconds to allow for the beads to evenly disperse the cells over the plate. The glass beads are then removed and the plates can be put in the incubator. This method was found to be significantly superior to the glass pipet method with minimal risk of contamination or damage to the cells.

To ensure that the colonies on plates were spread out and not too many colonies grew per plate, at least two different dilutions (usually 1x stock and one either 5 or 10 fold diluted) were plated. This ensured colonies were spread out enough so that they could be picked separately and were able to reach appropriate size for the blue/white screen (discussed below).
After 16 hours at 37 °C there should be colonies growing on the plate. Four colonies were picked individually and separately grown up in 25 mL of Luria broth with 50 μg/mL Tc in a 125 mL flask in a shaker at 37 °C. After another 16 hours, or when the cultures appeared to be densely grown up but not overgrown, 5 mL of each culture was spun down for a mini prep. Mini preps were performed using the Qiagen Spin Miniprep Kit (Cat. no. 27104). The final elution from the mini prep columns was performed with 50 μL of water, to maximize concentration. Elution buffer was not used, to avoid any additional cleaning step for sequencing.

Qiagen Spin MiniPrep Kit (Cat. no. 27104) Protocols:

1. Resuspend pelleted bacterial cells in 250 μL of Buffer P1 and transfer to a microcentrifuge tube. Buffer P1 contains 100 μg/mL RNaseA to breakdown RNA, 10 mM EDTA (to chelate divalent cations and thereby inhibit DNases), a pH dependent indicator dye, and 10 mM Tris-HCl, pH 8.0 as a buffer.

2. Add 250 μL of Buffer P2 and mix thoroughly by inverting the tube 4-6 times. Buffer P2 contains 200 mM NaOH and 1% sodium dodecyl sulfate (SDS) to lyse the cells. The tube will turn blue to indicate the solution is properly mixed. Do not allow this step to proceed longer than 5 minutes before adding Buffer N3 in step 3.

3. Add 350 μL of Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. Buffer N3 contains a 4.2 M Guanidine-HCl (Gu-HCl) and 0.9 M potassium acetate, pH 4.8, to neutralize the NaOH from Buffer P2. The blue color will disappear once Buffer P2 has been neutralized. A large white precipitate should be visible.

4. Centrifuge for 10 minutes at 13,000 rpm in a table-top microcentrifuge.

5. Apply the supernatant (from step 4) to the spin columns provided in the kit and centrifuge for 60 seconds. Discard flow-through.
6. Wash spin column with 0.5 mL of Buffer PB and centrifuge for 60 seconds. Buffer PB contains 5M Gu-HCl and 30% isopropanol. The Gu-HCl helps to shield the high charge of the DNA and therefore allows the DNA to bind tighter to the silicon-based matrix of the column. Discard flow-through.

7. Wash column with 0.75 mL of Buffer PE and centrifuge for 60 seconds. Buffer PE contains 80% ethanol and 10 mM Tris-HCl, pH 7.5 to aid in the binding of DNA to the column and to wash away chaotropic salts used earlier. Discard flow-through.

8. Centrifuge tube for 60 seconds again and then discard collection tube. Place column into clean collection tube.

9. To elute DNA, add 50 μL of double distilled H$_2$O to center of column material. Wait 2 minutes.

10. Centrifuge tube for 60 seconds and collect flow through.

To ensure that the correct plasmid was purified, a restriction digest was performed with EcoR I (New England Biolabs) followed by running the digest on a 1% agarose gel. The following general protocols were used for restriction digests:

- 9.5 μL H$_2$O
- 3 μL DNA
- 1.5 μL buffer (respective for restriction enzyme)
- 1 μL restriction enzyme
- 15 μL total

Add enzyme last and incubate at 37 °C for 1-2 hours.
Agarose gels were made in house and run using the following protocols:

1. Weigh out 1 g of electrophoresis grade agarose and dissolve in 100 mL of TBE
   1 L of TBE: 10.8 g of Tris base, 5.5 g boric acid, 4 mL of 0.5 M EDTA, dissolved in 1 L of water

2. Combine in 125 mL flask and microwave at 20 second increments until solution is boiling.
   Do not permit the solution to over boil. Remove from the microwave and stir on stir plate.

3. Aliquot 25 mL into each of 3 clean conical tubes for future gel making. These can be microwaved later and poured for making future gels. Pour final 25 mL into gel cast that has been taped on both ends with laboratory grade masking tape. Carefully place the eight-lane comb in the gel and make sure it is not too deep, otherwise the gel will leak.

4. Once the gel has solidified (polymerized), carefully remove the comb from the gel as not to damage the wells. Carefully remove the tape from the sides of the gel. Slowly remove the gel and glass plate together from the gel caster. Place the plate and gel into the electrophoresis apparatus. Be sure the wells are on the negative electrode side.

5. Add TBE to the electrophoresis apparatus so that the entire gel is submerged

6. Add appropriate amount of 6x loading dye (Promega: G190A) solution to each sample to be loaded onto gel (2.5 µL for 15 µL sample).

7. Add 5 µL of a kilobase ladder (New England Biolabs) to well #1 (Figure 28).

8. Add DNA samples of interest to wells #2-#8. If the DNA sample was from a digest, the entire sample was loaded. Maximum capacity is approximately 17 µL per well, depending on the depth of the comb when casting the gel.

9. Attach negative electrode from power supply onto well side of gel and positive electrode from power supply onto non-well side of gel. Turn on power supply to 125-150 V and run
for approximately 1.5-2 hours. The gel is finished running 5 minutes after the last loading
dye (blue) comes off the gel. The blue dye is bromophenol blue and migrates at
approximately 300 bp on a 1% agarose gel.

10. Once the gel is finished running, turn off the power supply and remove the electrodes from
the electrophoresis apparatus. Remove the gel from the electrophoresis apparatus and rinse
with distilled water. Put the gel in 0.5 μg/mL ethidium bromide solution for 10 minutes.
Ethidium bromide intercalates between the bases of the DNA and makes the DNA visible
under ultraviolet (UV) light. After 10 minutes pour the ethidium bromide back into the
stock container, rinse off the ethidium bromide with distilled water, and soak the gel in
distilled water for 5 minutes. The DNA should now be visible when the gel is viewed with
UV light on a gel box.

11. Take a photo of the gel using the Polaroid camera (Polaroid Spectra film: 74100 24242).

After confirming the restriction digest matches the expected DNA fragment lengths, the
plasmid needs to be sequenced to confirm that the mutation of interest is present. For mutations
at M265, primer M233RLa (a generous gift from Eiji Takahashi) or primer M144K was used for
sequencing. To quantitate the plasmid concentration the absorbance at 260 nm was measured
with an Agilent diode array spectrophotometer. An extinction coefficient of 50 mM⁻¹cm⁻¹ was
used to estimate the plasmid DNA concentration. Six (6) μL of plasmid (100-200 ng/μL) were
sent to the Core Sequencing Facility on the University of Illinois Urbana-Champaign campus.
The primers were also sent, at a concentration of 5.0-10.0 pm/μL, which converts to
approximately 125 ng/μL. If the plasmid concentration from the mini prep was too low, the
DNA was dried down under N₂ gas and resuspended in water to the proper concentration. It
usually took approximately 48 hours to get the sequence back from the sequencing facility.
Sequences were blasted (blast.ncbi.nlm.nih.gov) against the L and M subunit DNA (Figure 29) to check for the proper mutation. Plasmids were only accepted if the correct mutation was present and there were no other mutations or sequence anomalies. One of the most common problems with DNA sent for sequencing was that it came back with many unidentifiable nucleotides (Ns). When the DNA was resubmitted with an increased concentration it often came back with identifiable nucleotides.

Once a plasmid was identified with the correct mutation, six restriction digests were run in parallel, using EcoR I and the protocols listed above, to digest the plasmid. Digests were run for 1.5-2 hours to ensure complete digestion. The digested plasmids were then run on a 1% agarose gel using the protocols above. EcoR I digestion cuts the plasmid twice into two different bands: 4 kb and 3 kb. After the gel is stained and destained, the 4 kb band, which contains the puf operon, was cut out and the 3 kb band was left behind. The 4 kb is then transferred to the electroelution apparatus (IDT) and is placed on the DNA gel platform. The apparatus works the same way as an agarose gel, except that the DNA travels through the electroelution buffer and gets trapped in the tube next to the DNA gel platform. The small electroelution tube that connects the DNA platform to the opposite side of the apparatus is filled with 200 μL of 3 M sodium acetate, which acts as a salt trap. The salt trap shields the DNA from experiencing the electric current. Therefore the DNA will move until it enters the salt trap, where it will significantly slow or even come to a complete stop. The large reservoirs on each side of the apparatus are filled with electroelution buffer: 20 mM Tris, pH 8.0, 200 μM EDTA, and 5 mM NaCl. It is important that the reservoir level is high enough so that there is a continuous path of electroelution buffer from one reservoir through the DNA sample and salt trap well to the other reservoir. Up to six different samples can be run at the same time in the electroelution apparatus.
The positive (red) electrode is put on the opposite side of the DNA platform and the negative (black) electrode is placed on the same side as the DNA platform. Before the power supply is turned on, it is important to make sure the valve which connects the two reservoirs is closed so that the current runs through the DNA samples and the DNA trap. The three positions are completely depressed (all the way down): open between the two reservoirs; middle: closed between the two reservoirs; completely pulled up: open between two reservoirs and drain. Once the setup is complete, the voltage from the power supply is set to 120 V and turned on for 20 minutes. If the apparatus is running properly, there should be bubbles visible at the anode and cathode. After 20 minutes, the power supply is turned off and the control valve is pulled all the way up for draining of the reservoirs. The reservoirs are drained slowly as to not disturb the DNA trapped in the salt trap. Raising or lowering a tube attached to the drain spout can control the rate of drainage. Once the level of the reservoirs is below the level of the salt trap tube, drainage can be stopped. The sample from the salt trap tube is removed with a long thin pipet tip and placed in a microcentrifuge tube. Next, the volume removed from the salt trap tube is measured and 2.5 times this amount of 100% ethanol is added to the microcentrifuge tube for ethanol precipitation. This microcentrifuge tube is then put at -80 °C for at least 2 hours.

While digesting the pUC19 plasmid, 300 ng of the pRK415 plasmid is also digested. However, only 1 tube of pRK415 digestion is necessary for each mutant being cloned [4]. Following a 2 hour digestion, ethanol precipitation is performed by adding 1.5 μL of 3 M sodium acetate followed by 42 μL of 100% ethanol. This is stored in the -80°C for at least 2 hours and then follows the same procedure as the pUC19 plasmid below for DNA isolation from ethanol precipitation.
After at least 2 hours in the -80 °C freezer, the microcentrifuge tube from the ethanol precipitation is spun down in the cold room at maximum speed in a microcentrifuge (13,000 rpm) for 30 minutes. The ethanol is carefully decanted off so not to lose or disturb the pellet. The pellet, which may not be visible, is washed with 1 mL of -20 °C chilled 70% ethanol. This is spun down again for 15 minutes in the cold room at maximum speed in the microcentrifuge (13,000 rpm). The ethanol is decanted off again and the tube is left open to dry for 1 hour or can be quickly dried under nitrogen gas.

Once the ethanol has all evaporated, the cloning proceeds to the DNA ligation step. Eleven (11) μL of water are used to resuspend the ethanol precipitated pRK415 plasmid. This 11 μL is next transferred to the insert ethanol precipitation tube, which is the tube where the ligation will be performed. Next, 3 μL of 5x T4 DNA ligase buffer and 1 μL of T4 DNA ligase are added to the microcentrifuge tube. It is important that the buffer be thawed on ice as it contains ATP. Not thawing the ligase buffer on ice can lead to break down of the ATP to ADP and make the buffer function to a lower or non-functional capacity for the ligation assay. The ligation mixture is left at 4 °C for 16-48 hours to maximize the probability of ligation.

After 16-48 hours, half of the ligation mixture (7.5 μL) is used in a transformation of DH5α cells (see transformation protocols above). The cells are plated onto LB plates containing 15 μg/mL Tc, 50 μg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and 100 μM IPTG (isopropyl β-D-1-thiogalactopyranoside). DH5α cells are a broad host cell line that lacks the Lac operon so that they can be used for blue/white screens. A blue/white screen is so named because colonies that have an active form of β-galactosidase can cleave X-gal and the cleaved X-gal is visibly blue. Cells that cannot cleave X-gal stay white. IPTG is added to turn the Lac operon constitutively on. The pRK415 plasmid has a Lac operon and the EcoR I
cleavage interrupts this operon. Therefore, when the insert is present in pRK415, the colonies appear white ($\beta$-galactosidase is non-functional) because the Lac operon is interrupted. If pRK415 self anneals, the colonies appear blue ($\beta$-galactosidase function is intact) because the Lac operon is functional. These plates are put into a 37 °C incubator for 12-16 hours.

After colonies are visible on the plate, it is critical to make sure that colonies are large enough to be visibly blue and to make sure that the colonies are not growing on top of one another so that they can be independently picked. If all the colonies look white on the plate from a blue/white screen, the plate should be returned to the incubator and checked every 2 hours for colonies to turn blue. Once blue colonies are visible, 6 white colonies should be picked and cultured independently in 125 mL flasks with 25 mL of LB + 15 μg/mL Tc at 37 °C in the shaker. The remnants of the picked colonies should be checked periodically on the plate to make sure that they do not turn blue. If the colony turns blue, the corresponding culture should be discarded.

After approximately 12-16 hours the cultures will appear grown to an opaque density. If the cultures overgrow, discard them. Cultures are overgrown if cell clumps or precipitate is stuck to the sides of the flask. Five (5) mL of each culture are then minipreped using the Qiagen Spin Miniprep kit (protocols above). Three (3) μL of the minipreped plasmids are digested with Sph I (New England Bio Labs) for 1.5-2 hours at 37 °C. These digested samples are run on a 1% agarose gel (protocols above). Sph I cuts the pRK415 plasmid in three locations, making three separate pieces of DNA with lengths of 1.5, 6, and 7 kb. It is important that the bands on the gel be the correct lengths because these determine if the insert orientation is correct. If the orientation is reversed or there was some strange insertion, the bands will be off. If the band lengths are not correct in any of the lanes, then pick more white colonies or repeat the ligation
step. Alternatively, the second half of the ligation (remember only 7.5 μL were used) could be used for a transformation as well. If the correct orientation is present, the mutated region of the plasmid is sequenced, using the methods discussed earlier, to confirm that the mutation is still present and that there are no anomalies. The pRK415 plasmid is a low copy number plasmid and there are often only 1-2 plasmids per bacteria. Therefore it was necessary to modify the Qiagen Spin Miniprep protocols to take this into account.

Qiagen Spin MiniPrep kit (Cat. no. 27104) protocol modification for low copy plasmids:

1. Centrifuge down 4 tubes of 5 mL of the cultured bacteria (total of 20 mL) and discard supernatant.
2. Resuspend pelleted bacterial cells in each tube in 250 μL of Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250 μL of Buffer P2 to each tube and mix thoroughly by inverting the tube 4-6 times. The tube will turn blue to indicate the solution is properly mixed. Do not allow this step to proceed longer than 5 minutes before adding Buffer N3 in step 3.
4. Add 350 μL of Buffer N3 to each tube and mix immediately and thoroughly by inverting the tube 4-6 times. The blue color will disappear once Buffer 2 has been neutralized. A large white precipitate should be visible.
5. Centrifuge all tubes for 10 minutes at 13,000 rpm in a table-top microcentrifuge.
6. Apply the supernatant (from step 4) onto a single spin column provided in the Qiagen kit and centrifuge for 60 seconds. Discard flow-through. Repeat this process until the supernatant from all 4 tubes has been loaded onto one column and centrifuged. The column holds approximately 800 μL and therefore this step will need to be repeated approximately 4 times.
7. Wash spin column with 0.5 mL of Buffer PB and centrifuge for 60 seconds. Discard flow-through.

8. Wash column with 0.75 mL of Buffer PE and centrifuge for 60 seconds. Discard flow-through.

9. Centrifuge tube for 60 seconds again and then discard collection tube. Place column into clean collection tube.

10. To elute DNA, add 50 μL of double distilled H₂O to center of column material. Wait 2 minutes.

11. Centrifuge tube for 60 seconds and collect flow through.

With the correct orientation and sequence, it is now possible to proceed to the conjugation step. Conjugation is a process in which one bacterium is able to share a plasmid with another bacteria. The bacterium transferring the DNA is referred to as F+ indicating that it has the proper genetics to form a pilus to transfer the DNA. The bacterium receiving the DNA is known as F- simply for convenience sake, as it may also have the proper machinery to transfer DNA. The DNA is transferred via a mechanism called rolling circle replication.

Before conjugation can begin, the pRK415 plasmid must be put into a bacterium that is F+. S17-1 is a strain that is F+ and is used to conjugate the pRK415 plasmid to *Rb. sphaeroides* [5]. S17-1 carries a trimethoprim resistance gene. The *Rb. sphaeroides* strain, +KSS, has had the LH1, LH2, L and M genes knocked out from its genome and replaced, via a suicide vector, with a kanamycin resistance (Kam') gene. +KSS is used as the F- strain in this conjugation step.

The pRK415 plasmid is transformed into the S17-1 cell line by the heat shock methods described earlier and plated onto 50 μL/mL trimethoprim (Tm) and 15 μL/mL Tc LB agar plates.
A single colony is picked and cultured in 50 mL of LB with 50 μL/mL Tm and 15 μL/mL Tc in a 125 mL flask, at 37°C in a shaker, for conjugation. At the same time a +KSS cell culture is started in 50 mL of Sistrom’s minimal medium in a 125 mL flask with 25 μg/mL kanamycin (Kam) at room temperature (under 29°C). After 12-16 hours, the S17-1 culture should appear dense and opaque. As long as the S17-1 culture is not over grown, transfer 5 μL from the old culture to a 125 mL flask with 50 mL of LB, 50 μL/mL Tm, 15 μL/mL Tc, and culture the cells in an incubator at 37 °C. By the time the second culture of S17-1 is nearing opaqueness, the +KSS should also be dense enough for conjugation. If the +KSS is not yet entirely opaque or is still clear, reculture the S17-1 once more following the instructions above. The absorbance of both cell lines should be checked regularly. Once they reach 50 Klett units the cultures are ready for conjugation. It was found that the exact density of this last step was not critical. As long as both the S17-1 and the +KSS cells were in exponential growth and dense in appearance (but not overgrown), conjugation worked.

Spin down 1 mL of each cell line separately in a microcentrifuge tube in a bench top microcentrifuge at maximum speed (13,000 rpm) for 5 minutes. Pour off the supernatant and resuspend both cell lines in 1 mL of Sistrom’s. Spin down both tubes again for 5 minutes and pour off the supernatant. Resuspend the +KSS and S17-1 cells in 50 μL of Sistrom’s medium each. Pipet 5 μL of S17-1 cells into the 50 μL of +KSS cells. Pipet the entire 55 μL onto a Sistrom’s medium plate, allow to spread, and let dry. To make a Sistrom’s medium plate, add 20 grams of agar to 1 L of Sistrom’s medium before autoclaving, then follow the same instructions as for LB plates. Place the conjugation plate at 32°C for 6-8 hours in the dark, with the plate sitting right side up.
After 6 hours remove the plate, add 1 mL of Sistrom’s medium to the plate and scrape as much of the conjugation as possible into a microcentrifuge tube. Resuspend the cells with 1 mL of Sistrom’s medium in a microcentrifuge tube. Plate these cells onto Sistrom’s plates with 1.5 μg/mL Tc and 25 μg/mL Kam. Put these plates at room temperature (below 29°C) wrapped in foil sitting upside down. It will take approximately 5-7 days for colonies to be visible on the plates. Once brownish colored cells begin to appear, pick 3 colonies and culture them individually in 125 mL flasks in 50 mL of Sistrom’s with 1.5 μg/mL Tc and 25 μg/mL Kam. The cultures will likely take 3-5 days to begin growing. If the cultures begin growing immediately (less than 2 days), make fresh antibiotics and repeat the conjugation step. It is likely that there is a contaminant or that the antibiotics are bad.

Once the cultures have grown, make freezer stocks of every different *Rb. sphaeroides* mutant that grew. This is performed by mixing cultured cells with glycerol to a final concentration of 15% glycerol. Perform a mini prep using 20 mL of the culture to purify the pRK415 plasmid. Use the modified method for isolating low copy plasmids described above. Finally, sequence the L and M genes on these plasmids to confirm that the mutation of interest is present and no other anomalies exist in the RC genes. The primers listed in Figure 30 were used to sequence the L and M subunits in their entirety. The bacteria can now be cultured for RC purification.

Should the stocks become contaminated in the future and need to be restreaked onto plates, it is important that 5-10 colonies are picked and combined in the culture growth for the new stocks. This will protect from genetic drift.

**Growth of GA and R-26H Reaction Centers:**
GA and R-26H are two strains of *Rb. sphaeroides* commonly used to study the bacterial photosynthetic reaction center. Although there are genetic differences between the GA and R-26H strains, functionally they are quite similar. The major difference between them is that the R-26H strain reaction center lacks a carotenoid [6]. These strains are from the wild and therefore do not contain a His-tagged RC.

GA and R-26H were grown anaerobically photoheterotrophically in a modified Sistrom’s minimal media (the original is based on succinate and is better at suppressing non-sphaeroides contaminants like *E. coli*; the malate is cheaper).

Sistrom’s minimal medium (malate version):

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrated bases (see below)</td>
<td>240 mL</td>
</tr>
<tr>
<td>Growth factors (see below)</td>
<td>12 mL</td>
</tr>
<tr>
<td>DL-Malate</td>
<td>48.3 g</td>
</tr>
<tr>
<td>Potassium phosphate (dibasic)</td>
<td>41.8 g</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>6 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>6 g</td>
</tr>
<tr>
<td>KOH</td>
<td>approx. 33 g</td>
</tr>
</tbody>
</table>

Dissolve in H₂O. Adjust pH to 6.9.

Adjust final volume to 12 L with H₂O.

Concentrated bases:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrilotriacetic acid (NTA)</td>
<td>10 g</td>
</tr>
</tbody>
</table>
MgSO$_4$·7H$_2$O: 29 g 

Or MgSO$_4$ anhydrous: 14.6 g 

CaCl$_2$: 1.25 g 

Or CaCl$_2$·2H$_2$O: 1.7 g 

NH$_4$-molybdate·4H$_2$O: 9.2 mg 

FeSO$_4$·7H$_2$O: 200 mg 

Metals 44 (see below): 50 mL 

10% KOH: As needed 

Dissolve in H$_2$O. Adjust pH to 7.0. Adjust final volume to 1 L with H$_2$O. 

Metals 44: 

Makes 1 Liter: 

Free Acid EDTA*: 2.0 g 

Or tetra-sodium EDTA: 2.5 g 

Or di-sodium EDTA: 2.1 g 

ZnSO$_4$·7H$_2$O: 11.0 g 

FeSO$_4$·7H$_2$O: 5.0 g 

6N H$_2$SO$_4$: 1.5 mL 

MnSO$_4$·H$_2$O: 1.5 g 

CuSO$_4$·5H$_2$O: 0.4 g 

H$_3$BO$_3$: 0.12 g 

CoCl$_2$: 200 mg
Or CoCl$_4 \cdot 6$ H$_2$O 370 mg

*If free acid EDTA is used, omit H$_2$SO$_4$ and add 0.5-0.6 g NaOH

Dissolve in H$_2$O and adjust final volume with H$_2$O to 1 L.

Growth Factors (1000x):

Makes 200 mL:

biotin: 4 mg
niacin (nicotinic acid): 200 mg
thiamine-HCl: 100 mg
para-amino benzoic acid (PABA): 20 mg

Dissolve in H$_2$O & filter sterilize.

Starter cultures of bacteria were grown in 125 mL bottles of Sistrom’s medium. One mL of a freezer stock of the cells was added to the 125 mL bottle under flame using sterile technique. The freezer cell lines came from Eiji Takahashi who has been in the Wraight lab for many years. The bottle was completely filled up with Sistrom’s from another sterile bottle in order to eliminate air in the bottle. This is necessary to eventually create an anaerobic environment. The bottle was flamed and then sealed tightly with a screw top. R-26H cells require 12-24 hours of darkness before being put into light so that the bacteria consume all the oxygen present in the media. If this is not performed, growth of R-26H may be stagnant or the bacteria may not grow at all because previous studies have shown that lack of carotenoids leads to a lack of protection from oxygen free radical formation. The dark incubation is necessary for consumption of all of
the oxygen [6]. The bottles are kept in the dark by covering them with a black blanket or by placing the bottle into a drawer. GA cells do not require dark incubation and can be placed directly into the light following mixing of freezer stock and sterile media, because they have a carotenoid.

After dark incubation, the bottles were put under illumination from 25 W incandescent light bulbs, which surround the bottles in a laboratory built light bank. The bottles warm up due to the proximity of the incandescent bulbs (4-12 inches), and are kept below 29°C by fans continuously blowing ambient air over them. *Rb. sphaeroides* grows best at approximately 29°C. The starter cultures take 7-10 days to grow to a dense/opaque looking state. R-26H and GA cells look blue-green and green respectively.

Once the starters are grown, 10 mL of the culture was used to start each 1 L medicine bottle. Medicine bottles are used because they have a greater surface area to volume so that light can reach most if not all of the bacteria growing in the bottles, and the large surface area allows good heat exchange for keeping the bottles below 29°C. This large surface area allows the cultures to grow to higher densities than would be possible with a round bottle. These 1 L cultures are prepared under flame using sterile technique by pipetting 10 mL from the starter bottle into 1 L bottles containing autoclaved Sistrom’s medium. The bottles are then filled to the upper limit with Sistrom’s from another autoclaved bottle. This step is performed to limit the amount of air to which the culture is exposed, as was previously done with the 125 mL bottles. They were then capped with an airtight screw cap. These bottles were then placed in the dark for 12-24 hours before being put under illumination by 25 W incandescent light bulbs for R-26H growth, or immediately put under illumination for GA. The same light bank as described above for the 125 mL bottles was used for these bottles. After 7-10 days the bottles were harvested...
using the Millipore Pellicon filtration system and/or spun down in a centrifuge at 7000 x g in 250 mL bottles.

Alternatively, R-26H or GA can be grown in a 12 L carboy. The carboy is filled with 12 L of Sistrom’s medium and is autoclaved for 1 hour. Up to 3 carboys can be autoclaved at once. A magnetic stir bar was placed inside of each carboy prior to it being autoclaved. For 12 L carboys, a 1 L medicine bottle of grown culture was used to start 2 carboys (500 mL per carboy). Any airspace in the carboy was filled using autoclaved Sistrom’s medium from 1 L bottles. This was performed under flame using aseptic technique. The carboy was slightly overfilled to minimize air in the carboy after insertion of a stopper. The carboys are sealed with a rubber stopper that was previously autoclaved or soaked in ethanol prior to use. To reduce the air left in the carboy, a long needled syringe is inserted next to stopper during the stoppering. The air is removed through the syringe as the stopper is pushed in place. The goal is to leave no visible air bubbles in the carboy and to have an airtight seal. Aluminum foil is used to cover the black stopper so that under illumination the stopper does not act as a heat sink. As with the smaller cultures, R-26H carboys were kept in the dark for 12-24 hours before being put under continuous illumination from 25 W incandescent light bulbs 4 inches from the top of the carboy. GA was put under illumination immediately without dark incubation. To regulate temperature, the carboys were placed in a water bath with cold water from the tap running through a copper pipe in the bath to keep the bath cool, with fans blowing ambient air across the top of the carboys. The carboys are placed on top of a magnetic stir plate to spin the magnets inside of them. As the carboys are much thicker than the 125 mL bottles and medicine bottles, the stir bars aid in mixing the culture for even light exposure for photosynthetic growth and for heat exchange to
keep the culture homogeneous in temperature. All these practices combined keep the
temperature below 29 °C.

It takes between 7-10 days for the carboys to reach a deep opaque color. At this point the
cells maybe harvested. The bacteria were harvested with the Millipore Pellicon filtration system
and by centrifugation (7000 x g for 12 minutes in 250 mL bottles). Pellets were weighed and
frozen in the -80 °C freezer for later use. One 12 L carboy usually yields approximately 50-75 g
of cells.

The SMpHis cell line was grown in a similar fashion to GA and R-26H [7]. This cell line
expresses the WT RC with a Tc' gene. The starter cell culture for SMpHis was grown in the
light bank described above, in the presence of 1.5 μg/mL of Tc. Since tetracycline is light
sensitive, the starter culture bottles are wrapped in a red plastic filter to keep the Tc from
breaking down. Conveniently, the wavelengths that are let through are appropriate for the cells
to grow, but of low enough energy that they minimize Tc destruction. After the starter culture,
Tc is usually not added as it is expected that the large quantity of quickly growing SMpHis cells
will outpace any other contaminants from the starter. SMpHis cells can be grown in 12 L
carboys or 1 L bottles following the same procedure as for GA or R-26H listed above.

**Mutant Rb. sphaeroides Growth:**

There are a few problems that can occur in growing *Rb. sphaeroides* with mutant RCs.
The major problem with studying mutant reaction centers is a high risk that the mutant may
revert back to WT during growth. In order to prevent reversion, it is important to avoid any
environmental pressure that may encourage the bacteria to mutate back to their WT state. This is
accomplished by growing the mutants non-phototrophically. The main idea is that the mutants
are grown in a way in which the reaction center is not vital to energy production, but the RC is still expressed at a reasonable level.

Semi-aerobic non-photosynthetic growth is the answer to this problem. RC expression is controlled by light and the lack of oxygen, which turn on expression of the puf operon [8, 9]. The L and M subunits are on the puf operon, which is mainly regulated by oxygen concentration and operon is turned off in the presence of oxygen. Therefore, cells grown aerobically will have a minimal expression of RCs and cells grown anaerobically will have a significantly higher expression of RCs. To achieve a high concentration of RCs and limit pressures on the mutant bacteria to revert, the cells are initially grown aerobically to a high density and then undergo semi-aerobic growth, all in a dark nutrient rich environment. During semi-aerobic growth the cells are in a low oxygen environment, but not completely devoid of oxygen as in anaerobic growth. This leads to expression of the RC, but not the same levels as if the cells were grown photosynthetically. Therefore the RC yields per cell weight are lower compared to photosynthetically grown bacteria, but there is no pressure for reversion, as all energy is generated from the media.

The other major problem in growing mutants is contamination. There are many steps taken to ensure no contamination occurs, including stock growth in Sistrom’s minimal medium (no yeast extract) and the use of antibiotics.

To begin growth of any mutant strains or WT cells for that matter, it is important that a clean cell line and stock be available. To confirm cell lines are clean, they can be grown on Sistrom’s plates without antibiotics if no resistance genes are present, or with antibiotics if the cell line carries a resistance gene. The construct used for all the mutants discussed here has Kam’ and Tc’ genes. Another way to verify that a cell line is clean and not contaminated is to
grow a starter aerobically in Sistrom’s medium devoid of any yeast extract. It is possible to tell if there are bacteria of non-\textit{Rb. sphaeroides} origin, such as \textit{E. coli}, because the latter will grow significantly faster (within 1-2 days) than \textit{Rb. sphaeroides}. \textit{Rb. sphaeroides} will take 5-7 days to grow in Sistrom’s medium. This test can be used for both plates and liquid media.

The starter is grown in a pre-autoclaved 125 mL flask that is autoclaved empty with foil covering the top. The flask is autoclaved empty because the loss in volume of Sistrom’s from autoclaving would be significant making it difficult to add the appropriate amount of antibiotics. The Sistrom’s medium may also be more concentrated from the water losses during autoclaving and this could lead to various growth problems, reduced yields, higher risk of contamination, etc. Sistrom’s medium, autoclaved separately, is added to the flask using sterile technique. Twenty-five to fifty (25-50) mL of culture can be grown in the 125 mL flask. Antibiotics are then added if appropriate. For the mutant constructs discussed in this work, 1.5 \( \mu \text{g/mL} \) of Tc and 25 \( \mu \text{g/mL} \) of Kam are added to the starter. The flasks are grown in the dark in a shaker at room temperature (20-29 °C). The starter can be started with either a freezer stock from the -80 °C or colonies picked from plates. Growth usually takes 5-7 days from freezer stocks and approximately 4-7 days from a colony picked from a plate. Often it is possible to check visibly for contamination by observing growth that is significantly faster than 5 days (1-2 days), by seeing a change in color other than a white milky color, or by seeing clumps of cells or precipitate in the flasks. As it takes significantly longer to grow \textit{Rb. sphaeroides} than \textit{E. coli}, it is possible for mold or fungus to contaminate the cultures as well. Should any of the above occur in the starter culture, it should be discarded immediately and restarted with a fresh flask, fresh Sistrom’s, and a different cell stock. Since the cultures take so long to grow, it is often
advisable to start twice as many starters to ensure that at least one of them will not be contaminated.

Once clean cultures have grown to a good density that are not overgrown, the larger cultures for harvest can be grown. The next step is also an aerobic growth, but this time in Sistrom’s with 0.1% yeast extract added (Sistrom’s + 0.1% YE). This step is performed in 2 L flasks, which are autoclaved with 475 mL of the Sistrom’s + 0.1% YE, with an expected loss of approximately 25 mL after autoclaving for 45 minutes. The flasks are covered with a piece of foil large enough to cover the top of the flask and cover the side of the flask at least three inches down from the opening to reduce the risk of contamination. To these flasks is added 1 mL of starter culture, 1.5 μg/mL of Tc, and 25 μg/mL of Kam. The flasks are then put in shakers (New Brunswick series 25) for 2-4 days, shaking at 250 rpm. They are kept in the dark and grown at room temperature (20-29 °C). If the temperature goes above 30 °C, *Rb. sphaeroides* will have a difficult time growing. This is the aerobic phase of growth in which the interest is get the culture to a fast growing, high concentration of cells, but which are not overgrown or contaminated. The same criteria can be used for determining overgrowth or contamination as with the starter cultures, except that the color will be a milky yellow because of the added yeast extract. The aerobic growth phase of semi-aerobic growth is completed when the cultures have a nice milky yellow cell density with no visible precipitates or clumped cells. If precipitates form or there appears to be contamination, the culture is discarded. The density of the cells should be high enough that they are opaque. If there is no contamination and the cells have the appropriate color with good density, it is time to move to the semi-aerobic growth step.

The semi-aerobic growth step limits the amount of aerobic metabolism the cells will undergo and since they are not grown in light and cannot perform photosynthesis, it is important
to give the cells additional nutrients. To each 2 L flask, 1 L of Sistrom’s with 0.6% yeast extract is added, with 1.5 μg/mL of Tc for the 1 Liter. At this point it is no longer necessary to add Kam because the culture is so dense that it should be able to outcompete any other contaminants. However, it is crucial to add tetracycline to ensure the mutants do not lose their plasmid, which carries the mutation of interest and the Tc\(^r\) gene. The additions are performed using sterile technique and the flasks are put back in the shaker for 24 hours at 250 rpm. After 24 hours the shaker is slowed down to 200 rpms for 24 hours. Finally the shaker is slowed to 150 rpms for 24 hours. At this point flasks should look a dark brown or dark brown-green color. Some of the flasks may have precipitate, but at this point this is acceptable. If there are precipitates, one must take care during harvesting the cells not to pour the precipitates into the harvesting mixture, which goes into the Millipore Pellicon. The precipitates will clog the harvester. If precipitates are present, the flask is left sitting on the bench for 30 minutes so that the precipitates can fall to the bottom of the flask. Then only the top portion of the flask is poured into the harvesting solution. This can be repeated taking care to keep the precipitates in the flask. The final remaining few hundred mL in the flask are centrifuged rather than put into the harvester. Should the harvester become clogged, it is necessary to clean the harvester with Triton X-100 detergent, which can add an hour or more time to the harvesting procedure.

By following the steps above, the bacteria do not feel pressure to generate energy from the RCs and therefore do not revert. This allows RCs that are non-functional or semi-functional to be studied. After harvesting using the Millipore Pellicon cell harvester, the cells are centrifuged at 7,000 x g in 250 mL bottles and the supernatant is discarded. Pellets of cells are stored at -80 °C.
Reaction Center Purification:

When purifying non-His-tagged RCs, it is convenient to make chromatophores first when purifying reaction centers. Chromatophores are the membranes that contain the photosynthetic reaction centers, the \(bc_1\) complex, and the light harvesting antenna proteins. For His-tagged RCs, some of the chromatophore steps are skipped (see below; His-tagged Reaction Center Purification).

Non-His-Tagged RC purification:

Making Chromatophores (all work should be performed on ice and intense light should be avoided where possible):

1. Frozen cell pellets are stored at -80 °C and it will therefore take at least a few hours and preferably overnight to thaw the cell pellet. Add 150-200 mL of NET (100 mM NaCl, 0.5 mM EDTA, 10 mM Tris, pH 8.0) and a stir bar to the frozen cell pellet. Place the bottle on a stir plate in the cold room overnight. If there is a rush to thaw the cells, they can be thawed at room temperature on a stir plate and monitored regularly for thawing. Do not allow the pellet to thaw completely at room temperature. Once the pellet is mostly thawed it should be moved to the cold room to complete the thawing process. Before moving onto the next step, it is important to make sure the pellet is completely resuspended and that there are no solid clumps of cells floating around or sticking to the inside of the bottle.

2. Centrifuge the cells at 7,000 x g, for 20 minutes at 4° C. This step will wash the cell pellet of unwanted material. After centrifugation, inspect the supernatant and the pellet. The supernatant should be clear. If it is not, pour off the supernatant and resuspend the cells. Centrifuge the cells again and continue to repeat until the supernatant is clear. Once the supernatant is clear, weigh
the cell pellet and bottle. Remember to weigh the empty bottle later and subtract the bottle weight from the bottle and cell pellet weight to get the actual cell weight.

3. Resuspend the pellet in NET with approximately 160 mL of buffer per 50 g of cells.

4. Add a spatula tip of DNase and add MgCl$_2$ to the mixture to make approximately 1 mM MgCl$_2$. The Mg$^{2+}$ is needed to activate the DNase. (It was found that step 4 could be skipped without significant change to the final purity of the RCs, especially in His-tagged preps.)

5. French press the mixture at 16,000 psi. Add EDTA to the broken cells to bring the mixture to approximately 1 mM EDTA. This is performed to chelate Mg$^{2+}$ and Ca $^{2+}$ to inhibit proteases from chewing up the RCs. (Addition of EDTA at this step is not necessary if step 4 above was skipped.)

6. Centrifuge the crushed cells at 27,000 x g for 20 minutes to sediment unbroken cells and large debris. If a large pellet is present, resuspend in NET and repeat the French press step.

7. Spin the supernatant in the ultracentrifuge at 225,000 x g for 1.5-2 hours. It is important to check the ultracentrifuge tubes and make sure there are no cracks that may leak during the ultracentrifugation step. If the tubes leak, sample will be lost and damage could be caused to the ultracentrifuge vacuum pump.

8. Resuspend the pellets in a small volume of NET buffer (2-3 mL per pellet), using a small paint brush. Avoid resuspending any grey-blue (R-26H) or green-yellow (GA) core of the pellet.

9. Combine the resuspended pellets and let them stir overnight in the cold room or for as long as possible for same day usage. The solution should contain chromatophores which can be frozen at -80 °C for later use.

**Purifying Reaction Centers from Chromatophores for non-His-tagged RCs:**
1. Measure the near infrared (NIR) absorbance spectrum of chromatophores (750-950 nm), using 950 nm as a baseline. Determine $A_{802}$ and dilute the chromatophore stock to $A_{802}=50$ with NET buffer.

2. Add 30% stock Lauryldimethylamine-N-oxide (LDAO) dropwise in the dark (cover beaker with foil) while stirring at room temperature (25 °C), to a final concentration of 0.6%.

3. Continue to stir mixture for 60 minutes at 4 °C, in the dark.

4. Spin in the ultracentrifuge at 225,000 x g for 1.5-2 hours. Be sure tubes are full and top up with 0.6% LDAO in NET buffer.

5. Keep the supernatant. Measure the NIR absorbance. Determine absorbance at 802 nm and use 950 nm as a baseline. The concentration of RCs can be calculated using the Beer-Lambert law [10]:

$$A = \varepsilon CL$$  \hspace{1cm} (A = \text{absorbance (unit less)}, \varepsilon = \text{extinction coefficient (mM}^{-1}\text{cm}^{-1}), C = \text{concentration (mM)}, L = \text{path length (cm)}; \text{the extinction coefficient for RC at 802 nm } \varepsilon_{802} = 288 \text{ mM}^{-1}\text{cm}^{-1}.$$

Multiplying the concentration by volume of RCs gives the total RCs:

$$C \times V = Y$$  \hspace{1cm} (C = \text{concentration (mM)}, V = \text{volume (mL)}, Y = \text{Yield of RCs (μmoles)}

Expected yield at this point is 500 nmoles per 25 grams of wet cell pellet.

**Ammonium Sulfate (AS) Precipitation:**

1. Add 0.4% LDAO to make the final concentration 1.0%.

2. Slowly add solid AS to a concentration of 0.27 g/mL while stirring. Then stir for 20 additional minutes.

3. Centrifuge the sample at 27,000 x g for 10 minutes.

4. Resuspend the levitate (the portion that floats on top of the supernatant) in AS 125 buffer (AS Buffer= 0.5 g/mL AS adjusted to pH 8.0 with NaOH; TL1= 1.0% LDAO in NET; AS 125
buffer= 0.125 g/mL of AS, but made by mixing 1 part AS buffer and 3 parts TL1.) This is best done using a glass syringe and sucking up the levitate up and down through the needle. Caution needs to be taken to avoid causing any bubbles as this could denature the RCs. Once resuspended, stir for 15 additional minutes at room temperature.

5. Centrifuge at 15,000 rpm for 10 minutes and keep the supernatant for later use.

6. Reextract the pellet with AS125 buffer and centrifuge at 15,000 rpm for 10 minutes. Combine this supernatant with the supernatant from step 5.

Measure the volume of the combined supernatants and add ½ volume of AS buffer dropwise. This will give a final AS concentration of 0.25 g/mL.

7. Centrifuge sample at 15,000 rpm for 10 minutes. Resuspend the levitate in Teal8 (Teal8: 10 mM, Tris pH 8.0, 0.1 mM EDTA, 0.1% LDAO).

DEAE Protocols for FPLC (these protocols can also be performed with a gravity column):

1. Set up the FPLC so that two 5 mL DEAE columns (GE Biosciences) in series are selected and equilibrate them with Teal8. For Buffer A place a liter of Teal8 and for Buffer B place a liter of Teal8 with 1 M NaCl. Make sure that the concentration of B is set to 0%.

2. Motorized valve 1 (injection valve) is set to load (position 1). Using pump C, load the superloop. The superloop should be loaded at a rate of 15 mL/min (any faster than this can introduce air into the system). Shut off pump C when the superloop is almost full (approx. 45 mL). Take precautions not to over load the superloop as it can crack.

3. Load the column by selecting motorized valve 1 and setting it to inject. Turn the flow rate up on pump A/B to 3 mL/min. A greater flow rate than this could damage or shorten the lifetime of the column. Repeat steps 2 and 3 until the entire sample is loaded or the bottom column is starting to turn green from protein entering it. It is recommended to leave at least
half of the bottom column empty to allow for creeping of the protein during purification. When the superloop is almost empty turn off pump A. Overrunning the superloop during loading of the DEAE columns will not damage the superloop, but could introduce air into the system.

4. Once your sample is completely loaded onto the column rinse the column with 40 mL of Teal8. To do this turn on pump A to 2-3 mL/min and continue to run it until 40 mL of Teal8 has washed the column.

5A. At this point rinse the column with higher salt. Set the concentration of pump B to 8% (equivalent to 80 mM NaCl) and rinse with 40 mL. This wash step can be done at 2 mL/min flow speed.

5B. Wash the column with 100 mM NaCl (10% pump B concentration) for 40 mL.

5C. Finally, wash the column with 120 mM NaCl (12% pump B concentration) for 40 mL.

6. Elute the RCs with 250 mM NaCl (25% concentration B) at 2 mL/min. Collect the green flow through in one beaker. Once the green flow through has been collected dilute it 4x with Teal8. Wash the DEAE columns with 100% pump B concentration (1 M NaCl) for 40 mL or until the columns look clean.

7. Repeat steps 1-5 of FPLC purification. However, this time elute at 180 mM NaCl (18% pump B concentration) at a flow rate of 0.5 mL/min. It is likely that this elution will take many hours (5-6 hours). Set the fraction collector to collect fractions of 1.25-1.4 mL. Program the computer to turn the fraction collector on after 20 mL of elution to make sure that there are enough tubes for collection.

8. Check which fractions have RCs in them by looking at how dark green each sample. Next run spectra on every 10th fraction to determine where the cleanest RCs are found. Make sure
the peak at 850 nm is much lower than the peak at 802 nm for the RCs. If the peak at 850 nm is larger than the 802 nm peak, light harvesting pigments are present and need to be removed. If the peak at 850 nm is still greater than or equal in amplitude to the 802 nm peak, then repeat step 7. If the peak is small, compare the ratio of $A_{280}/A_{802}$; any fraction with a ratio better than 1.3 should be kept. The rest of the fractions that have RCs but not at a high enough purity should be grouped and run through the 250 mM NaCl purification on the FPLC.

9. Centricon the samples that are kept, to concentrate down to a volume suitable for dialysis.

10. Dialyse with Teal8 buffer overnight, changing the buffer at least twice.

11. After dialysis, the sample is stored at -80 °C for future use unless the sample is to be used for crystallography. In this situation, the sample is not frozen, but kept on ice for immediate crystal tray setting.

**Flash Spectroscopy:**

Flash spectroscopy is an optical kinetic spectroscopic technique with the added feature of a flash lamp to excite optically active samples, such as the RC. The flash spectrophotometer in the Wraight lab is one that was originally built by Colin A. Wraight, with recent software improvements by Olexandr (Sasha) Kokhan. A 12 V car headlight lamp provides the measuring beam of light. Next is a monochromator, which dissects the wavelengths of light from the headlight to single wavelengths. This light then passes through a shutter and onto the sample housed in a cuvette. The signal is finally detected by a photomultiplier tube. At a normal plane to the detection beam is the optics for a xenon flash lamp, used to excite the primary donor of the RC. The flash lamp operates at approximately 1200 V. This device allows the user to study
electron transfer reactions in the RC in the forward and reverse directions at various wavelengths to elucidate the kinetics and thermodynamics of the system. The software that runs the shutter, photomultiplier, and flash lamp is controlled by a program written with Labview by Sasha Kokhan.

RCs were kinetically characterized by optical flash spectroscopy on the kinetic spectrophotometer described above. The kinetics of charge recombination in the absence of secondary quinone (Q$_B$) ($k^{A}_{p}: P^+Q_A \rightarrow PQ_A$) were measured by monitoring the disappearance of P$^+$ at 430 nm in the presence of 50 μM terbutryn, which blocks Q$_B^-$ formation [11]. Rates for $k^{A}_{p}$ were on the order of 10 s$^{-1}$ and therefore were measured generally using the following details:

- Time Points: 0.1 μsec
- Length of Trace: 5000 points
- Over Sampling Ratio: 1600
- Total Time: 0.8 sec
- Time Constant: 30 kHz

Charge recombination in the presence of Q$_B$ ($k^{B}_{p}: P^+Q_AQ_B \rightarrow PQ_AQ_B$) was also measured at 430 nm by following P$^+$ disappearance, but in place of terbutryn, 20 μM Q-10 was added to reconstitute Q$_B$ maximally. Rates for $k^{B}_{p}$ were on the order of 0.1-1 s$^{-1}$ and therefore were measured with the following settings:

- Time Points: 1 msec
- Length of Trace: 5000 points
- Over Sampling Ratio: 10-1600
- Total Time: 5-150 sec
- Time Constant: 30 kHz
First electron transfer kinetics ($k_{AB}^{(1)}$: $P^*Q_AQ_B \rightarrow P^*Q_AQ_B$) were measured at 397 nm, which measures the electrochromic shift of the bacteriopheophytin in response to $Q_A^-$. 20 μM Q-10 was added to maximize the presence of quinone in the $Q_B$ site. $k_{AB}^{(1)}$ was in the range of 500-10,000 s$^{-1}$ and was measured with the following settings:

- Time Points: 0.25 μsec
- Length of Trace: 5000 points
- Over Sampling Ratio: 1
- Total Time: 12.5-25 msec
- Time Constant: 1 MHz

Second electron transfer kinetics ($k_{AB}^{(2)}$: $P^*Q_AQ_B \rightarrow P^*Q_AQ_BH_2$) were measured by the decay of the semiquinone signal at 450 nm which is indicative of the proton coupled second electron transfer. 1-200 μM ferrocene were added to reduce $P^*$ and 0.5-1 mM ferrocyanide to reduce the ferrocenium byproduct. Rate constants for the 2nd electron transfer ($k_{AB}^{(2)}$) were in the range of 50,000-5 s$^{-1}$, depending on the mutant. The following parameters were used to measure the 2nd electron transfer kinetics:

- Time Points: 0.5 μsec
- Length of Trace: 5000 points
- Over Sampling Ratio: 25-200
- Total Time: 0.5 sec
- Time Constant: 300 kHz

pH titrations were performed starting at pH 7.0 and either going up to pH 11.0 or down to pH 4.0 by increments of 0.5 pH units. All samples that were used for $Q_A^-$ back reaction, $Q_B^-$ back
reaction, 1st electron transfer, and second electron transfer kinetic pH titrations had the following buffer conditions:

1-1.5 μM RCs (1st electron transfer 4 μM RCs)

0.02% Triton X-100

2.5 mM KCl

1 mM succinate

1 mM citrate

1 mM Mes

1 mM Mops

1 mM Tricine

1 mM Ches

1 mM Caps

pH was adjusted using 0.1-1 M HCl or 0.1-1M NaOH

Reversibility of the pH titration was tested by measuring a few points back to pH 7.0 after reaching either pH 4.0 or pH 11.0.

The data was imported and processed using the computation software Origin.

References:


Figures:

Figure 27: The map of the pUC19 plasmid (courtesy of New England Biolabs)
Figure 28: Kilobase ladder from New England Biolabs: catalog # N3232S.
Figure 29: L and M subunits combined:

1 aceggagagg gaagcatgge actgctcagc ttcgagcgaa aatatcgcgt gccgggggc
61 acgctgtgct gcggaaacct gttcgacttc tgggtgcgcc ctttctatgt cggcttcttc
121 gggtttgcga cgtttttctt gcggaaacct gttcgacttc tgggtgcgcc ctgggaggtg cacccctcgc aaaaggcggg ctgtggcaga tcatcacgat ctgcgccact
181 ctccagggta cctggaaccc ccaactcact ctgtgctacc cggcggcctg ggtatcattc tgattgcctg gagtgccgta
241 cttgggaggtg cactcctcgc gtcgctcttc cggcggcctg ggtatcattc tgattgcctg gagtgccgta
301 ggttccttcg tcagctgggc gctgcgcgaa gtcgaaatct gccgtaagct gggcatcggg
taccacatee cgttgccttc cggcggcctg ggtatcattc tgattgcctg gagtgccgta
361 taccacatee cgttgccttc cggcggcctg ggtatcattc tgattgcctg gagtgccgta
421 cgccgggtga tgatgggcgc ctggggctat gccttcccct acgggatctg gaagcatcctc
gactgggtgt cgctcggccc ggtatcattc tgattgcctg gagtgccgta
481 atcggaacttc accttcggct cggcgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
gccgttcgcc atcctggcct acctgacgc
t ggtgctgttc
481 gctgggccat ggcaagcaca cegcgctggc gtctctctcg ggcattgatggt
gctgggcttc atccgtccga tcctcatggg gtcctggtcg gaagcggttc cctacggcat
cctctacac ctcgactgga cgaacaactt ctgcctgctg caacggcaacc tgtttctaca
cctcttccg gggtctcctc tcgcttcctc taaggggtcg gcccttctct ctcgatgca
cggtgcacc accatctgctg ttccccgtt cggcggcgc agcagatgc
ggacaccgggc aegcagcgag aegggccgcg cctctctg ggtgttctgg egctggacca tgtttcga
cgaccagag gcagctggcc catctggatg gcgtctctc tctgagctac
cgacacggc ttcggtggc aacctga
gggtctcctg ctctggggca gaaccacggc atggcgccgc tgaactga

**Figure 29 (cont):** L & M subunits. The two subunits slightly overlap, which is why the M subunit does not start at nucleotide #1 [12, 13].
Sequencing primers:

M\textsubscript{rev}: 5’ cgg att ggt gtt gag gtg atc g 3’

M\textsubscript{233RLa}: 5’ ctt cgg cgg cga gct cga gct gga gca g 3’

M\textsubscript{136RQ}: 5’ ggc cgc acc tat ctg cag gct cag ggc ctg 3’

M\textsubscript{4Q}: 5’ atg cct gag tat cag aac atc ttc tcc cag 3’

M\textsubscript{34Pb}: 5’ gag cag ggt cga gaa ggg acc gac gcc cga 3’

M\textsubscript{144K}: 5’ ctg ggc atg ggc aag cac acc gcc tgg ggc 3’

L\textsubscript{5F}: 5’ gca ctg ctc agc ttc gag cga aaa tat cgc 3’

L\textsubscript{139M}: 5’ cgc cgc ggg atg atg gcc gtc ttc ttc agc 3’

L\textsubscript{239S}: 5’ ctg ctc tgc agc gcc gtc ttc ttc agc 3’

**Figure 30:** Sequencing primers: Primers M\textsubscript{rev}, M\textsubscript{233 RLa} and M\textsubscript{136 RQ} were generous gifts from Eiji Takahashi. Primers M\textsubscript{4Q}, M\textsubscript{34Pb}, M\textsubscript{144K}, L\textsubscript{5F}, L\textsubscript{139M}, and L\textsubscript{239S} were ordered from IDT. Primers M\textsubscript{rev} and M\textsubscript{34Pb} are anti-parallel to the L & M genes and therefore are used to sequence the genes backwards. All other primers sequenced in the same direction that the gene is transcribed.