Characterization of Photosynthetic Reaction Centers from Bradyrhizobium Strain BTAi 1

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CHARACTERIZATION OF PHOTOSYNTHETIC REACTION CENTERS FROM BRADYRHIZOBIUM STRAIN BTAi 1

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

Isaac Paul Forquer
B.S., Wright State University, 1998
WRIGHT STATE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

August 12,2002

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Isaac Paul Forquer ENTITLED Characterization of Photosynthetic Reaction Centers from Bradyrhizobium strain BTAi 1 BE ACCEPTED IN PARTIAL FULLFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Abstract

Forquer, Isaac Paul. M.S. Department of Biochemistry and Molecular Biology, Wright State University, 2005. Characterization of Photosynthetic Reaction Centers from Bradyrhizobium strain BTAi 1

Photosynthetic rhizobia have been studied for about 15 years now. They are now considered to be metabolically aligned with a relatively recently discovered group of bacteria, the anoxygenic aerobic phototrophs (AAP’s). Rhizobia form symbiotic relationships with plants from the Fabaceae family. Photosynthetic rhizobia not only nodulate the roots, as most other rhizobia do, but they also form nodules on the stems of certain leguminous plants. The plant provides carbon to the bacteria and the bacteria provides the plant with soluble nitrogen fixed from the biologically inert but abundant atmospheric N₂.

A key question regarding photosynthetic rhizobia and other AAP’s derives from the observation that photosynthesis in these organisms shuts down under anaerobic conditions. It has been proposed, and is the hypothesis of this thesis that the primary electron acceptor (QA) in the photosynthetic reaction center has a higher midpoint potential than in reaction centers found in the AAP’s counterparts, the anaerobic purple bacteria. If QA had a higher midpoint potential, it would be more labile to overreduction under anoxic conditions, and if QA is reduced, then photosynthetic electron transport is blocked.

A redox titration was done to measure the midpoint potential of QA in the reaction centers of BTAi 1. This was done by observing the level of P (primary electron donor) bleaching
upon excitation with bright light at different ambient redox potentials. The level of P
bleaching is proportional to the fraction of QA that is not reduced, since P cannot bleach
and donate an electron if QA is already reduced.
Reaction centers from BTAi 1 were purified using two techniques, both involving ion
exchange chromatography and one involving ammonium sulfate precipitation. Reaction
centers were characterized by spectrophotometric studies, mass spectroscopy studies
(MALDI TOF) and the cofactor composition was determined.
The midpoint potential of QA in BTAi 1 is –44 mV vs. SHE. The molecular weights of
the subunits are very comparable to other photosynthetic reaction centers, from both
aerobic and anaerobic bacteria. The pigment stoichiometry of reaction centers from
BTAi 1 is 2:1 bacteriochlorophyll:bacteriopheophytin. Both absorbance and light minus
dark absorbance spectra are nearly identical to that found in anaerobic photosynthetic
bacteria.
Photosynthetic reaction centers in BTAi 1 are very similar to reaction centers of
anaerobic photosynthetic bacteria. The midpoint potential of QA cannot account for its
overreduction under anaerobic conditions. It is likely that AAP’s lack a key enzyme that
would participate in redox homeostasis of the photosynthetic electron transport chain.
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**Introduction**

*Overview:*

Photosynthesis is the fundamental process used by phototrophic organisms to convert solar light energy into biochemical energy in the form of ATP and reducing equivalents such as NADPH. Photosynthetic organisms include plants, algae, some protists, cyanobacteria and photosynthetic bacteria. Plants, algae, protists and cyanobacteria all use the same general scheme for photosynthesis, in which two reaction centers (or photosystems) are used to perform non-cyclic photosynthesis. Photosynthetic bacteria use one reaction center in a cyclic electron transport pathway (Blankenship & Hartman, 1998) (see fig.1 for general models of both)

---

**Fig. 1: Models for bacterial and higher organism photosynthesis**
Bacterial photosynthesis has another profound difference from the more sophisticated plants, algae, protists and cyanobacteria. Photosynthetic bacteria are *anoxygenic*, which is to say that the primary light reactions do not evolve oxygen, as do the higher photosynthetic organisms. This also indicates that photosynthetic bacteria do not use water as the source for electrons in photosynthetic electron transport. Rather, the photosynthetic bacteria ultimately derive electrons for electron transport from either organic acids such as malate and glutamate, or other reduced compounds such as inorganic sulfur (H₂S etc.), methanol and hydrogen (Madigan, 1991).

Photosynthetic bacteria have been known by science for at least 50 years. Until the early 1980’s, all of the photosynthetic bacteria known photosynthesized under anaerobic conditions only. Some of the bacteria studied were able to live and grow aerobically, but used oxidative metabolism under such conditions. Four major groups are used to describe these bacteria from a taxonomic standpoint. The purple non-sulfur, the

---

**Fig 2.** X-ray crystal structures of reaction centers from *Rhodopseudomonas viridis* (left), and *Rhodobacter sphaeroides* (right). Note that only *R. viridis* contains the tetra-heme cytochrome c subunit. These structures were derived from the PDB coordinates 1PRC (*R. viridis*) and 1AJ (Rh. sphaeroides).
purple sulfur, the green non-sulfur and the green sulfur groups. The reaction center found in purple bacteria is thought to be the evolutionary origin of PSII in plants, while PSI is thought to be derived from the green bacteria (Blankenship, 1992).

Photosynthetic reaction centers are large integral membrane proteins weighing around 100 kDa (some have a bound tetra-heme cytochrome, which adds 40 kDa)(Roy, 2001). The reaction centers from two purple non-sulfur bacteria are to date the most comprehensively described reaction centers in the literature.

The first large integral membrane protein to have its crystal structure solved to atomic resolution was the reaction center from *Rhodopseudomonas viridis*. Michel, Diesenhoffer and Huber shared the Nobel Prize in chemistry in 1988 for their efforts in completing this enormous and important task (Deisenhofer & Michel, 1989). Later, the reaction center from *Rhodobacter sphaeroides* was crystallized and its atomic resolution structure is now solved as well (Ermler et al., 1994). The *R. viridis* reaction center contains four subunits (L, M, H and C). The L and M subunits are membrane-spanning and each contains five α-helices. The H subunit sits on top of the membrane-spanning subunits. It is mostly water soluble, and has one α-helix that spans the membrane to act as an anchor. The C subunit is a water-soluble tetraheme c-type cytochrome, and has no membrane spanning moieties. The *R. sphaeroides* crystal structure revealed three subunits (L, M and H). These three subunits were quite similar in structure and function to the corresponding subunits in the *R. viridis* reaction center. The big difference between the two species is the absence of the C subunit in *Rb. sphaeroides*. Figure 2 shows the x-ray crystal structures of the two reaction centers solved to atomic resolution.
The designation of L, M and H as subunit names is derived from some of the first SDS-PAGE experiments on purified reaction centers. Three subunits were resolved, and they were named Light, Medium and Heavy based on the order in which they migrated through the gel. With the advent of nucleotide sequencing, it was discovered that these names were actually misnomers. The L and M subunits each weigh more than the H subunit. The discrepancy was due to the fact that membrane proteins bind up to 5 times as much SDS as do water-soluble proteins, so their charge density will be greater and they will migrate farther down a gel than a protein with a near-equivalent molecular weight.

Almost every purple reaction center studied to date has the same stoichiometry of pigments and quinones. Each reaction center contains four bacteriochlorophylls, two bacteriopheophytins, two quinones and one iron atom. Table 1 lists the subunit and cofactor composition for the two crystallized reaction centers.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Bchl $a$</th>
<th>Bchl $b$</th>
<th>BΦ $a$</th>
<th>BΦ $b$</th>
<th>UQ</th>
<th>MQ</th>
<th>Hemes</th>
<th>Iron atom</th>
<th>Subunits</th>
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<tr>
<td>$R.\ viridis$</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>LMHC</td>
</tr>
<tr>
<td>$Rb.\ sphaeroides$</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>LMH</td>
</tr>
</tbody>
</table>


Figure 3 shows the 3-dimensional distribution of the various cofactors in each of the reaction centers. Note the striking similarity in spatial distribution of the cofactors. Also notice a symmetry associated with the distribution of the cofactors. This is
deceiving because only one side of the reaction center participates in electron transport. That is to say that one bacteriochlorophyll and one bacteriopheophytin do not actively participate in electron transport. Functional aspects of the cofactors will be discussed in the next section.

Fig. 3A

Figure 3. Arrangement of cofactors in photosynthetic reaction centers. P = “special pair” bacteriochlorophyll dimer; B = accessory bacteriochlorophyll; Phe = bacteriopheophytin; QA = Primary electron acceptor; QB = Final Electron acceptor. *R. viridis* (left) contains bacteriochlorophyll b and bacteriopheophytin b. *Rb. sphaeroides* (right) contains bacteriochlorophyll a and bacteriopheophytin a. Qa is menaquinone in *R. viridis* and ubiquinone in *Rb. sphaeroides.*

A: Three-dimensional distribution of cofactors (the two solid lines indicate the boundary of the lipid bilayer).
B: Structural details of the cofactors (except heme groups)

**Catalytic Cycle for the Photosynthetic Reaction Center**

The photochemical reactions that take place in the reaction center take place quickly and efficiently. Under ideal conditions, the quantum yield for reaction center catalysis can approach unity (Ke, 2001). Figure 4 shows the general scheme of electron
transport in the reaction center. For this description, the *Rb. spaeroides* structure is used, and so the activity of a bound cytochrome is neglected.

The photosynthetic reaction center is surrounded by antenna complexes called light-harvesting proteins. These proteins each span the membrane twice and each has two bacteriochlorophyll molecules and a bound carotenoid. The light-harvesting proteins capture most of the light used by the reaction centers, which is transferred to the special pair (P) bacteriochlorophyll dimer in the form of excitons. Once P receives the excitation energy from the light harvesting proteins, it undergoes an electronic transition into a singlet excited state. The electron is transferred extremely fast to B, and then to bacteriopheophytin. Bacteriopheophytin then transfers the electron to the QA quinone, which is the first stable electron acceptor. QA then passes its electron to QB, which is originally bound as an oxidized quinone and assumes the semiquinone form. Once P is re-reduced, the cycle starts over, ultimately leading to another reduction of the QB quinone (ubiquinone). Once QB is doubly reduced, it is stabilized by being doubly protonated and is then released to the quinone pool as a quinol:

\[
\text{Eqn. 1}
\]

\[
\text{Ubiquinone-n} \xrightarrow{2e^- + 2H^+} \text{Ubiquinol-n}
\]
After P donates its electron to the electron transport chain, it is re-reduced by a soluble c-type cytochrome. In the case of *Rb. spaeroides*, the soluble cytochrome donates its electron directly to P. In *R. viridis*, the cytochrome donates its electron to the bound tetraheme cytochrome. The bound cytochrome then donates the electron to P.

Interestingly, only one side of the reaction center is used for electron transport. One bacteriochlorophyll and one bacteriopheophytin are not used directly in electron transport. Reasons for their existence is speculative. One popular hypothesis is that they are not disposable due to structural considerations (Kirmaier *et al.*, 2003).

**Expanded View of the Electron Transport Chain**

Once Q_B is doubly reduced, it is released into the lipid bilayer as a quinol and the Q_B binding pocket of the reaction center is re-occupied by an oxidized quinone. The released quinol diffuses to the Q_O site of the cytochrome b/c_1 complex. Here one electron
is transferred sequentially to a Fe-S complex and cytochrome $c_1$. Cytochrome $c_1$ then donates it’s electron to cytochrome $c$, which will be released from the cytochrome $b/c_1$ complex and diffuse to the reaction center where it will re-reduce P. The second electron from the quinone in the $Q_o$ site is transferred to a low potential heme ($b_L$), then to a high potential heme ($b_H$). A second quinone-binding site on the cytochrome $b/c_1$ called $Q_i$ is right next to $b_H$, and it binds oxidized quinones. The quinone receives the electron from $b_H$ and is reduced, but as in the case of the reaction center, it requires the enzyme to turnover twice in order to doubly reduce the quinone to quinol and so the $Q_o$ site must oxidize two quinols in order for $Q_i$ to receive two electrons. The activity at the $Q_o$ also includes releasing the two protons from the quinol hydroxyl groups to the periplasmic side of the membrane. The $Q_i$ site, in doubly reducing its quinol gets its protons from the cytoplasmic side of the membrane, as in the $Q_B$ site in the reaction center. This cycling of electrons and protons is collectively called the “modified Q-cycle”, which most investigators accept as the mechanism for cytochrome $b/c_1$ complex activity (Crofts, 2000; Crofts, 2004; Trumpower, 1990). Figure 5 shows a diagram of the Q cycle. If one assumes that one turnover of the cytochrome $b/c_1$ complex is defined by oxidizing two quinones at the $Q_o$ site, the net proton movement can be described by Equation 2:

$$2QH_2 (Q_o \text{ site}) + Q (Q_i \text{ site}) + 2H^+ (\text{cytoplasm}) \Rightarrow 2Q + QH_2 + 4H^+ (\text{periplasm})$$

Where $QH_2 = \text{ubiquinol}$ and $Q = \text{ubiquinone}$
It should be noted the catalytic cycle described is simplistic. Many other energy-transducing proteins are present on the membranes of photosynthetic bacteria. Homologues of Complexes I (NADH-quinone oxidoreductase), II (succinate dehydrogenase), III (cytochrome $b/c_1$ complex), IV (cytochrome oxidases) and V (ATP synthase) are all found in photosynthetic bacteria. It is also quite possible that Complex I for instance plays a role in redox homeostasis of the quinone pool of \textit{Rb. sphaeroides} via reversed electron flow or some other alternative pathways (Tichi & Tabita, 2001; Tichi et al., 2001).

\textit{Organism Description:}
Photosynthetic bacteria can be found throughout nature. Almost every habitat known contains photosynthetic bacteria (Madigan, 1991). They have been found at all depths of the ocean and in almost every terrestrial setting. As mentioned earlier, the photosynthetic bacteria studied to date are almost exclusively anaerobic. Only in the last 20 years have bacteria that photosynthesize under aerobic conditions been known (Okamura et al., 1986). None of these bacteria can perform photosynthesis under anaerobic conditions. The reason for this is not yet understood.

Bradyrhizobium Strain BTAi 1 is a member of the group of bacteria known as the Rhizobiaceae (Fleischman & Kramer, 1998). Rhizobia form symbiotic associations with plants in the Fabaceae family in which the plant provides carbon to the rhizobium and the rhizobium provides soluble, fixed nitrogen to the host plant. Normally, rhizobia infect tissues in the roots of the host plant to form nodules, where they fix N₂ (Madigan, 1991). BTAi 1 and a number of other recently discovered rhizobia form nodules on the stems of certain leguminous plants as well as the roots (Fleischman & Kramer, 1998).

BTAi 1 is a member of a relatively recently discovered group of rhizobia. The extraordinary characteristic of this group is the fact that while they do fix nitrogen and form stem nodules on the host plants, they also contain bacteriochlorophyll. Bacteriochlorophyll was found in BTAi 1 in 1990 by Evans et al. (Eaglesham, 1990; Evans, 1990). Since then over 200 other rhizobia that form stem nodules and contain bacteriochlorophyll have been discovered. Reaction centers in photosynthetic rhizobia can undergo light-induced charge separation (P⁺ Q⁻), but only under aerobic conditions (Kramer et al., 1997). This fact aligns BTAi 1 with the relatively recently discovered aerobic anoxygenic phototrophs (AAP’s). The observation that BTAi 1 and other AAP’s
cannot photosynthesize under anaerobic conditions has led to debate on what is different about these bacteria. The most popular hypothesis in the literature is that the mid-point potential ($E_m$) of the QA quinone is higher than in anaerobic bacteria (Yurkov & Beatty, 1998). This would indicate that QA could more easily become overreduced and block electron transport in the reaction center. To be perfectly clear, QA is definitely being overreduced. Overreduction of QA can be indirectly observed by watching the optical density (OD) at 870 nm, which is the wavelength of light that reduced P absorbs. P bleaches when a flash of light is given, but it will only bleach if QA is available to receive and stabilize the electron from P, which is to say that P will only bleach if QA is in the oxidized form. The main question then asks if the overreduction of QA is an inherent property of the quinone and its binding pocket, or does a chemically different quinone species occupy the QA site or do AAP’s lack some unknown ability their anaerobic counterparts have to maintain the redox poise of the quinone pool.

Motivation for Research

The group of rhizobia known to photosynthesize represents an exciting prospect for improvements in third world agriculture. Industrially produced chemical fertilizers are cost-prohibitive for most third world farming communities. Chemical fertilizers also have the disadvantage of being associated with water and soil pollution, as in when lakes become eutrophic after an application of fertilizer on nearby land.

Biological nitrogen fixation can be challenging to manage and implement in settings where fields must be flooded for the plant to grow, as in rice cultivation. Root-nodulating legumes cannot be used in this situation because the oxygen tension would be too low to fuel respiration in the rhizobia. Alternatives to the root-nodulating rhizobia
are stem-nodulating rhizobia. These bacteria can form nodules on the stems of the plant, above the water line, where atmospheric oxygen would be available to the bacteria.

In addition to being able to nodulate stems, some stem-nodulating rhizobia can also photosynthesize. Photosynthesis may be a way for the rhizobia to enhance survival ex planta, promote stem nodulation, and may even contribute to the extremely energy-expensive process of biological nitrogen fixation. For every two moles of ammonia produced by rhizobia, 18 moles of ATP are consumed. Having a photosynthetic contribution to ATP synthesis could potentially alleviate the carbon stress that rhizobia impose on their host plants, and could contribute ATP directly to dinitrogenase activity.

**Hypothesis**

The original hypothesis of this thesis was that the QA quinone in BTAi 1 has a higher mid-point potential than is found in the anaerobic photosynthetic bacteria. In order to test this hypothesis, the first questions asked were concerning the general structure and organization of the reaction center:

1.) Is the stoichiometry of pigments the same? (bacteriopheophytin:bacteriochlorophyll)

2.) Is the distribution of subunits the same? (number and molecular weight of subunits)

3.) How does the primary structure of the polypeptides compare with R. sphaeroides and R. viridis? (are there significant differences in amino acid sequences, particularly around the QA binding pocket?)

4.) What is the mid-point potential of QA in this species? (How does it compare with the anaerobic photosynthetic bacteria?).
5.) What is the quinone species found in the QA binding pocket?

There are a few exceptional cases where the stoichiometry of the pigments arranged in the reaction center are different from the common rule of 2 bacteriochlorophyll : 1 bacteriopheophytin. *Chloroflexus auranticus* has a 1:1 ratio for instance (Ke, 2001). Based on the 16s ribosomal sequence derived phylogenetic trees, any indication of a close association with *C. auranticus* would have been quite surprising. A 2:1 ratio is the expected result, based on the similarity of major characteristics found in BTAi 1 with anaerobic phototrophic bacteria (spectra of membranes etc.).

Two possible outcomes would be expected with respect to the distribution of the subunits. One would be that the reaction centers would have the same distribution of subunits as *R. sphaeroides*, the other result expected would be that it were similar to that of *R. viridis*. Although this will be interesting data with respect to the general knowledge of the system, neither result will give evidence for a possible mechanism of over reduction.

The primary structure of the subunits would be the first really good and specific evidence if something were really different about the QA binding pocket. The mid-point potential of an electron carrier will greatly be affected by small changes in the immediate environment (10Å radius). Differences in the predicted dielectric constant of the binding pocket would, for instance, be interesting.

Measuring the mid-point potential of QA is clearly the best way to make assumptions about QA’s role in the observed overreduction. There are conflicting data in the literature concerning the mid-point potential of QA in other AAP’s. Some data
suggests that QA does have a higher mid-point potential in AAP’s than in the anaerobes, while other data suggest they are quite similar.

A difference in the actual quinone species found in the QA binding pocket would be yet another way to explain any difference found in the $E_m$ of the quinone. There is some precedent for finding different quinones, for instance *R. viridis* and *Rb. sphaeroides* do not have common QA quinones. They have menaquinone-$n$ and ubiquinone-$n$ in their QA binding pockets respectively. Any result that would indicate that BTAi 1 does not have one of the former quinones as its QA would be extremely interesting and would almost certainly predict a different $E_m$. 
**Materials and Methods**

*Growth Conditions for BTAi 1:*

BTAi 1 is grown in a defined minimal salts medium formulated by W.R. Evans (Evans’ medium). Appendix A lists the components and their concentrations in Evans’ medium. BTAi 1 from streaked slants on 1.5% agar and Evans’ medium were inoculated into small, ~200 mL Erlenmeyer flasks (seed cultures). The cultures were agitated on a laboratory shaker (~30 rpm). The cultures were also exposed to cyclic light (16h light, 8 h dark) from a 100 W incandescent bulb suspended approximately 1 M above the shaker. Once the seed cultures turned pink, they were used to inoculate 3 L Furnbach flasks containing Evans’ medium. The 3 L cultures were grown under cyclic (16h light, 8 h dark) white light and were stirred via a magnetic stir bar (~120-180 rpm). Once the culture turned pink, it was kept in the dark or under cyclic red light. After the culture reached late log phase, the bacteria were harvested.

*Preparation of Photosynthetic Membranes from BTAi 1:*

Cells were harvested by centrifugation in 250 mL bottles for 20 min at 5000 rpm in a Sorvall® SL-250 rotor. The cells were then washed once in buffer containing 50 mM
Tris·HCl, pH = 7.8. The washed cells were then exposed to sonication in 15 one-minute intervals with mixing between intervals. The broken cells were then centrifuged for 30 min in 50 mL tubes at 5000 rpm in a Sorvall® SL-50 rotor to remove large debris. The supernatant was decanted and saved. If the pellet still appeared intensely colored, it was resuspended and the sonication procedure was repeated until a mostly white pellet resulted.

The resulting supernatant from sonications was applied to a sucrose density step gradient (22%-55% w/w). Density gradients were necessary because of a resulting gummy substance that would make the chromatophores impossible to resuspend otherwise. The gradients were spun on a Beckman® ultracentrifuge (SW-50 rotor) for 1

![Figure 6. Typical absorption spectrum of BTAi 1 chromatophores. The 877 nm absorption band is primarily due to light-harvesting bacteriochlorophyll, while the 800 nm absorption band is due primarily to reaction center bacteriochlorophyll. The absorption bands to the blue of the bacteriochlorophyll bands are mostly from carotenoids. Y-axis units are Absorbance Units (AU)
hour at ~90,000 x g. A very intense red-purple band was collected at the interface of the two sucrose layers. The supernatant appeared orange-red and was collected and saved for cursory experiments.

Photosynthetic membranes (chromatophores) were analyzed on a Hewlett-Packard diode-array spectrophotometer for qualitative appearance and relative abundance of light harvesting proteins and reaction centers. Two major absorption bands were of interest in the absorption spectra of chromatophores, the 800 nm band (due to reaction center absorption) and the 877 nm band (due to light-harvesting bacteriochlorophyll with a slight contribution from reaction centers). See Figure 6 for a typical absorption

![Figure 7. A typical light-dark difference spectrum taken with chromatophores of BTAi 1. The major features are the 800 nm band-shift to the blue and the bleaching of the 870 nm band, which are both due to reaction center photochemistry. In addition, the bleaching of the “Q,” transition of the bacteriochlorophyll can be visualized at 600 nm. The bleaching at roughly 550 nm is due to cytochrome oxidation by P in the reaction center. The x-axis is wavelength (nm) and the y-axis is Δabsorbance.](image-url)
spectrum of chromatophores.

In addition to the absorption spectrum, a “light minus dark” spectrum was collected for the chromatophores on the diode-array spectrophotometer as well. In order to collect a light minus dark spectrum, a cuvette containing chromatophores was illuminated with saturating white light while an absorbance spectrum was collected. A “dark” absorbance spectrum collected with the same cuvette was then subtracted from the “light” spectrum. This light-dark spectrum will indicate if the reaction centers are viable, and can also indicate relative activity. If reaction centers are in good working order, a blue shift should be seen at 800 nm and a bleaching should be seen at 870 nm. See Figure 7 for a typical light minus dark difference spectrum of chromatophores from BTAi 1.

The collected chromatophores were then washed once by suspending in buffer containing 50 mM Tris·HCl, pH = 7.8 and ultracentrifuged for one hour at 90,000g. The washed chromatophores were stored at 4°C anaerobically in 10 mL volumetric flasks. Absorbance and light minus dark spectra were taken of the preps right before experiments to ensure that the reaction centers were active.

Estimation of the Redox Potential of the QA Quinone in BTAi 1 Reaction Centers (the following experiment was carried out in the laboratory of Dr. David Kramer at Washington State University)

In order to assess the redox potential of QA in photosynthetic reaction centers from BTAi 1, the fraction of P (870 nm band) that will bleach upon excitation is measured. P will bleach if QA is oxidized, and will not bleach if it is reduced because if the QA quinone is reduced it cannot accept an electron from P. Therefore, the relative
amplitude of the 870 nm band that will bleach upon illumination can be interpreted as the ratio of oxidized to reduced QA. Chromatophores were suspended in an anaerobic cuvette specifically designed for redox titrations. See Figure 8 for a schematic drawing of a typical cuvette used in redox titrations.

![Figure 8. Schematic diagram of a cuvette used for redox titrations. (Used with permission from Antony Crofts).](image)

The cuvette has multiple ports that allow for an argon inlet, exhaust, a salt bridge port, a stirring apparatus, an injection port and an inlet for the platinum electrode. The cuvette containing chromatophores in 50 mM Tris·HCl, pH = 7.8 was filled to a level sufficient to submerge the platinum electrode, salt bridge and stirrer. The cuvette was flushed with argon and stirred from 30 min before the experiment and through the entire experiment to ensure that the system was entirely anaerobic. In order to ensure that the bulk aqueous phase ambient potential was equilibrated with QA, redox mediators were used. See Table 2 for a list of the mediators used, their $E_m$ and their $n$-value ($n$= number of electrons involved in the redox reaction undergone by the mediator). Mediators with
an n-value of 1 have a useful mediation range of 60 mV above and below their $E_m$, while
n=2 mediators have a useful range of 30 mV above and below their $E_m$.

<table>
<thead>
<tr>
<th><strong>MEDIATOR</strong></th>
<th>$E_m$, n= 1 or 2</th>
<th><strong>Final Concentration used</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2 napthoquinone</td>
<td>+135 mV, n = 2</td>
<td>10 µM</td>
</tr>
<tr>
<td>Phenazine methosulfate</td>
<td>+80 mV, n = 1</td>
<td>5 µM</td>
</tr>
<tr>
<td>Phenazine ethosulfate</td>
<td>+55 mV, n = 1</td>
<td>5 µM</td>
</tr>
<tr>
<td>Duroquinone</td>
<td>+5 mV, n = 2</td>
<td>10 µM</td>
</tr>
<tr>
<td>2-hydroxy-1,4-napthoquinone</td>
<td>-135 mV, n = 2</td>
<td>10 µM</td>
</tr>
</tbody>
</table>

A home-built spectrophotometer was used for the experiment. The spectrophotometer is referred to as DOFS, or Diffuse Optics Focusing Spectrophotometer (see (Sacksteder et al., 2000) for a description of DOFS). Briefly, DOFS has a central chamber that is lined with an extremely reflective material. At one end of the chamber is a xenon flash lamp which provides pulses of measuring light. On either side of the chamber is a hole that opens to the sample and the reference cuvettes in such a manner that will distribute light of equal intensity and quality to both cuvettes. The light passes through blocking filters for wavelength selection, through the sample and reference cuvettes, and finally the light is passed through a compound parabolic concentrator that will focus an optimal amount of measuring light to the photodiode.
Exciting light is supplied to the experimental cuvette perpendicular to the measuring beam. A Q-switched laser was used as the excitation source. The laser beam had a wavelength of ~600 nm and was emitted in 8 ns pulses. The first 870 nm absorption band bleaching measurement was taken 8 ms after excitation. Subsequent measurements were taken to monitor if P was being completely re-reduced. See Figure 9 for a typical trace collected on the DOFS instrument.

The reference electrode in this experiment was a Ag/AgCl electrode (+221 mV vs. SHE). The reference electrode was bridged to the cuvette by a salt bridge (4 M KCl in 4% agar).

Figure 9. An example of a data point taken during the redox titration experiment. Four flashes were given. The amplitude after the second excitation was collected and compared to trials at different $E_a$'s.
For each measurement, the ambient potential, or $E_h$, was set by the addition of 20 µL of either 10 mM potassium ferricyanide or 10 mM sodium dithionite. Once the potassium ferricyanide or sodium dithionate was added, the system was allowed 30-40 min to reach equilibrium. While developing the assay, it was found that measurements taken before 30 min would result in hysteresis, resulting in erroneous estimations of the mid-point potential of QA. Once the $E_h$ in the bulk aqueous phase reached equilibrium with QA, the bleaching experiment was carried out. During the experiment, a repeatable, second deflection occurred at a potential higher than +100 mV. The absolute absorbance change observed at potentials where the curve appeared to be flattening before the high potential deflection was set as full scale. Subsequent trials at lower potentials were done and the absorbance change was normalized to full scale. The $E_h$ at which P bleaching was 50% of full scale was taken to be the mid-point potential of QA. The experiment was done “in both directions”, meaning that the potential was set to full scale, and aliquots of sodium dithionate were added, and once bleaching completely disappeared, aliquots of potassium ferricyanide were added until full scale was reached again. This was done to ensure that no hysteresis has taken place and to make sure the entire system is in equilibrium. For a detailed review of redox potentiometry see (Dutton & Wilson, 1974).

Different $n$-values ($n$ represents the number of electrons involved in a given redox reaction, in this case the transfer of electrons from mediator to QA) were substituted in the theoretical Nernst equations that best fit the data to test the hypothesis that one electron was involved in the reaction.
The data from the redox titration were plotted in Microsoft Excel® and the second derivative test was used to find the point of inflection in the graph. This was taken to be the estimation of the midpoint potential. This value was then substituted for \( E_m \) in the Nernst equation. Once a theoretical Nernst equation was developed, the line was superimposed on the data to test if the line behaved in a way that was true to the data.

**Identification of Amino Acids Present in the Q\(_a\) Binding Pocket of the Reaction Center**

The amino acid sequence of the L and M subunits from the reaction centers of *Rb. sphaeroides*, *R. viridis*, *R. denitrificans* and the *Bradyrhizobia* strain ORS 278 (Giraud *et al.*, 2000)(a phylogenetically close species to BTAi 1) were aligned using the Wisconsin® GCG package on the World Wide Web driven BioNavigator® software. Simple pairwise alignment functions were used. Amino acids within 10 Å of C-6 of the Q\(_a\) ubiquinone in the crystal structure of *Rb. sphaeroides* reaction center (PDB accession 1AIJ) were identified in the sequence alignment and compared to the other species.

**Development of an Isolation and Purification Procedure for Reaction Centers From BTAi 1.**

As a starting point, the methods recently developed by (Yurkov & Beatty, 1998) to isolate reaction centers from other aerobic photosynthetic bacteria were tested for the isolation of reaction centers from BTAi 1. Briefly, “LHI-RC particles” (partially purified membrane protein complexes) with an OD of 5 at 870 nm were treated with the
detergent LDAO (v/v) 2.5% (dimethylldodecylamine-N-oxide) and incubated for 2.5 hrs at 37 °C. This mixture was applied directly to a DEAE-agarose column, and the reaction centers were stripped from the column with a linear gradient of salt (0-500 mM NaCl). See the results section of the reference for a more detailed description of the purification procedure.

This procedure was not sufficient to purify reaction centers from BTAi 1. See the Results section for a description of the various problems encountered. Modifications of the procedure are outlined below.

The temperature was reduced to room temperature, and subsequently the incubation period was made longer. The activity of the reaction centers (estimated by light-dark spectral shifts) is lost during incubation with high concentrations of LDAO (2.5% or higher).

The loss in activity upon solubilization led us to experiment with replacing LDAO with Triton X-100 during the chromatography in order to eliminate the detergent as a source of the observed loss in activity. LDAO was kept in the procedure up until chromatography. The mobile phase of the column was made to be 20 mM sodium phosphate, pH = 8.00 and 0.1% Triton X-100.

As might be expected from the photochemical nature of reaction centers, turning all lights out and performing all operations in the dark (except a green filtered light source, as there is no absorbance in that part of the spectrum in any of the reaction centers thought to be similar to those of BTAi 1) was found to maintain activity post-chromatography. All steps carried out after treatment with detergent were carried out in either dim white or green filtered light.
The last major modification was diluting the chromatophores to an OD of 0.5 (instead of 5.0) at 870 nm.

A working procedure was found with the use of Triton X-100 as the detergent in the mobile phase during chromatography. The procedure is as follows:

1.) Chromatophores were isolated as described.

2.) The chromatophores were diluted to an OD = 0.5 at 870 nm. A 50 mL aliquot of this suspension was used.

3.) The suspension was made to be 2.5% LDAO (v/v) and incubated for at least 3 hours at room temperature.

4.) The mixture was centrifuged at 90,000 g for 1 hour to remove LDAO-insoluble material and any remaining cell wall material.

5.) The supernatant from the centrifugation was decanted, and mixed with LDAO again (to a final concentration of 5% (v/v) to ensure complete solubilization).

6.) The mixture was then applied to a DEAE Sepharose® column (20 cm X 1.5 cm). Once the sample was loaded, it was washed on the column with at least two column volumes. The mobile phase was 20 mM NaPO₄, pH = 8.00 and Triton X-100 (0.1%)

7.) Once washing was complete, a linear gradient of NaCl (0-500 mM NaCl) was applied to the column. Reaction centers came off the column with bacteriochlorophyll-containing light harvesting proteins at roughly 100 mM NaCl. The elution was repeated until all of the light harvesting proteins were removed (observed spectrophotometrically).
8.) The reaction center-containing elution bands were then ammonium sulfate precipitated to increase purity.

9.) The samples were analyzed spectrophotometrically and tested for purity. Unfortunately, Triton X-100 absorbs light very strongly at 280 nm, so the traditional 280/800 ratio could not be measured, as the Triton X-100 in the buffer had an unmeasurable absorbance at 280 nm.

The purification steps seemed to give good, working reaction centers, but without the 280/800 ratio, a quick and reliable estimation of the real purity of the samples was unavailable. Attempts at removing the Triton X-100 and exchanging LDAO back were unsuccessful.

Another procedure was attempted in which LDAO was the only detergent used. This method is inspired by the procedure outlined in (Wraight, 1979).

1.) The chromatophores were isolated as described above.

2.) The chromatophores were diluted to a final O.D. of 0.5 at 870 nm.

3.) The suspension of chromatophores was made to be 3.0% LDAO and allowed incubate for 2 hours at room temperature.

4.) The mixture was ultracentrifuged for one hour at 100,000 g.

5.) The supernatant was collected and the pellet discarded.

6.) The supernatant was fractionated with ammonium sulfate, three fraction were taken, 35%, 60% and 80% of saturation with ammonium sulfate.

7.) Each of the fractions resulted in pellets which floated. Each pellet was collected and analyzed spectrophotometrically.
8.) Ammonium sulfate fractions determined to be reaction center-enriched were applied to a DEAE Sepharose column.

9.) The sample was washed on the column with 2 column volumes of 20 mM NaPO₄, pH = 8.0 0.1% LDAO.

10.) The samples were re-eluted until an optimal 280nm/800nm was observed. (<3.0)

Because the OD at 280 nm could be measured in these samples, a simple scoring index was invented to track not only purity but also activity. The ratio of the OD difference between 790 nm and 810 nm in the light minus dark spectra of each sample was divided by 280 nm:

\[
\frac{\Delta \text{Abs } 790–810 \text{ nm}}{\text{Abs } 280 \text{ nm}}
\]

Alternative attempts at testing purity were SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) and MALDI TOF (Matrix Assisted Light Desorption Ion Time Of Flight) mass spectroscopy.

**SDS-PAGE And MALDI TOF**

Numerous procedures were tested in light of many problems encountered during attempts at resolving the subunits of the reaction centers. Initially, a 12% resolving gel was used. The proteins (10 ug protein) were treated with a denaturant containing SDS and β-mercaptoethanol with temperatures ranging from room temperature to boiling. Various treatment times were used to try and find an optimal denaturing condition.
Either the proteins did not enter the gel entirely or they did and smeared badly. Finally, the system was changed to a 15% resolving gel containing urea. This was found to resolve protein bands in a much more repeatable manner.

On every occasion that a gel was run, bands with higher apparent molecular weights than that of the hypothesized molecular weights of the reaction center subunits were found.

Again, different temperatures, different incubation times were used, and all gave different results. The addition of dithiothreitol instead of β-mercaptoethanol was employed to account for potential re-oxidation problems. The samples were treated with iodoacetamide in order to protect –SH groups during boiling. Dithiothreitol was added after boiling to try to re-reduce any disulfide bridges that may have been formed during denaturation at higher temperatures. None of these trials gave usable results.

Subsequent experiments with a MALDI TOF spectrometer (Model PBS II, from Ciphergen©) indicated that the higher molecular weight bands were not representative of single polypeptides. Rather there appeared to be aggregation of smaller polypeptides giving unreasonably higher apparent molecular weights. For this reason, MALDI TOF was used to estimate the molecular weight of the proteins present. Figure 10 shows a typical MALDI TOF spectrum of a sample prep.

The procedure for MALDI TOF is as follows:

1.) “Hydrophobic” chips were washed with acetonitrile and allowed to dry

2.) 1 µL of buffer containing the protein was applied to a spot on the chip, which was bordered with a hydrophobic pen to ensure spot integrity. 2 ng of protein was added to the chip.
3.) Once the buffer was completely evaporated, the light-absorbing matrix (sinapinic acid) was washed and spotted on top of the protein.

4.) The spot was analyzed by the MALDI TOF spectrometer.

5.) On some occasions, the protein sample acquired a +2 charge, rather than a +1 charge, which results in the data that indicate molecular weights half the real value. To overcome the false readings from a +2 charge phenomenon, the molecular weight data were multiplied by 2, and taken to be correct.

Multiple spots on each chip were run to ensure repeatability. The presence of a particular peak on three or more spots was taken to be a real result.

Determining the Presence or Absence of a Cytochrome c Subunit on the Reaction Center

In order to detect whether or not reaction centers found in BTAi 1 contained a permanently bound cytochrome c subunit, the TMBZ (3,3′,5,5′-tetramethylbenzidine) method [Thomas, 1976 #41] of cytochrome c detection was employed. Briefly, an SDS-PAGE gel is run as described earlier. Upon completion of electrophoresis, the gel is washed for 5 minutes. The washed gel is then incubated in 10 mL 0.5 mg/mL TMBZ in ethanol plus 23 mL of 250 mM sodium acetate, pH 5.00 for 30 minutes in the dark. At the end of 30 minutes, the gel is developed with 100 µL 37% hydrogen peroxide. The gel is allowed to develop until green bands appear, when the reaction is stopped by the addition of isopropanol.

Estimation of the Stoichiometry of Pigments Bound in the Reaction Center.
Approximately 100 µg of purified reaction centers were filtered on a Microcon©-30 filter. The protein was then resuspended in a bacteriochlorophyll-extracting solvent (7:2 v:v) acetone : methanol. Crystals of anhydrous magnesium sulfate were added to desiccate the sample. The extraction was allowed to incubate at room temperature for 30 minutes in the dark. After incubation, the extract was centrifuged to remove any light-scattering material, and the supernatant was saved for spectroscopic examination.

The sample was analyzed on a Hewlett Packard Diode Array spectrophotometer. The absorbances at 770 nm and 747 nm were collected. Using published (Straley & Clayton, 1973; Straley et al., 1973) extinction coefficients for bacteriochlorophyll and bacteriopheophytin, we estimated the ratio of bacteriochlorophyll to bacteriopheophytin using simultaneous equations. See Table 3 for extinction coefficients used. According to the Beer-Lambert law:

\[
A = \varepsilon Cl \quad \text{(equation 5)}
\]

<table>
<thead>
<tr>
<th>Pigment</th>
<th>Extinction coefficient at 770 nm</th>
<th>Extinction coefficient at 747 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriochlorophyll</td>
<td>76.0 mM(^{-1})·cm(^{-1})</td>
<td>52.6 mM(^{-1})·cm(^{-1})</td>
</tr>
<tr>
<td>Baceteriopheophytin</td>
<td>19.6 mM(^{-1})·cm(^{-1})</td>
<td>46.3 mM(^{-1})·cm(^{-1})</td>
</tr>
</tbody>
</table>

Table 3. Extinction coefficients of bacteriochlorophyll and bacteriopheophytin. From (Straley & Clayton, 1973; Straley et al., 1973)
From the given extinction coefficients, simultaneous equations can be written in the form:

\[ A_{770} = \left( \epsilon_{Bchl} \right)_{770} (1 cm) + \left( \epsilon_{Bphee} \right)_{770} (1 cm) \]  \hspace{1cm} \text{equation 6} \\
\[ A_{747} = \left( \epsilon_{Bchl} \right)_{747} (1 cm) + \left( \epsilon_{Bphee} \right)_{747} (1 cm) \]  \hspace{1cm} \text{equation 7} \\

Because the units of the extinction coefficient are mM\(^{-1}\)· cm\(^{-1}\) the light path value of 1 cm can be dropped, and the equations can be solved for the concentration of bacteriochlorophyll (Beja et al.):

\[ [\text{Bchl}] = \frac{\left[ \left( \epsilon_{Bphee} \right)_{770} (A_{747}) \right] - A_{770}}{\left( \epsilon_{Bphee} \right)_{770} - \epsilon_{Bchl}_{770}} \]  \hspace{1cm} \text{equation 8} \\

Once the concentration of bacteriochlorophyll is solved, the bacteriopheophytin can easily be calculated from either equation 6 or equation 7.

Attempts at the Qualitative Identification of the QA Quinone Species.

Reaction center preps were filtered and concentrated to 5-10 mg/mL. The protein was then suspended in a 1:1 mixture of acetone : methanol. The extraction took place at 37 °C for one hour. After the hour, the mixture was allowed to cool and then centrifuged at 12,000 rpm on an Eppendorf® microfuge to remove protein and other insoluble matter. One to two mL hexane was then layered on top of the acetone : methanol mixture, mixed, and allowed to sit for 5-10 minutes. The hexane was then decanted and allowed to evaporate to dryness. Once dry, 50 μL more of hexane was added. The entire 50 μL was then spotted on a pre-dried, activated silica gel plate for thin layer chromatography. The plate also contained standards of ubiquinone-10, and two menaquinone standards. The
best mobile phase constructed for the standards was 88:12 petroleum ether: acetone. Upon completion, the plate was allowed to dry, and was then sprayed with a reduced methylene blue solution to reveal ubiquinones and menaquinones.

The methylene blue solution was prepared by making a 1% solution of methylene blue in ethanol. To that 2–5g of zinc dust plus 2-3 mL concentrated sulfuric acid were added 10 minutes before use. The mixture was allowed to stand until it turned colorless, when it was filtered in a Buchner funnel to remove the zinc dust. The solution stayed clear for approximately 10 minutes. When the solution is sprayed on the TLC plate, ubiquinones react immediately and turn blue, whereas menaquinones, with their lower redox potential, react in about 5–10 minutes, turning green.

Because of multiple failures in this experiment, the TLC plates were treated with different reagents as well, including spraying with 50% concentrated sulfuric acid and heating on a hot plate for 1 hr, which digests all lipids. Also, the plates were sprayed with Rhodamine G 250, dried and viewed under an ultraviolet light source.
Results

Estimation of the Mid-Point Potential of $Q_A$

The measured midpoint potential of $Q_A$ in reaction centers from BTAi 1 was $-44$ mV. During the experiment, a high potential component of bleaching was found at potentials greater than $+50$ mV. This was probably due to the redox activity of cytochrome $c$, which is responsible for re-reducing $P^+$ (oxidized, or bleached special pair) in the reaction center. Accumulation of $P^+$ under conditions when the pool of cytochrome $c$ is relatively more oxidized is expected. Very similar values for $E_m$ were found when doing the titration in both directions. Figure 10 shows the titration. The experiment was repeated using a red LED for 4 seconds as an excitation source, and no difference was found in the $E_m$.

Some difficulties were encountered at potentials around $-50$ mV, and this is attributed to a

![Figure 10. Redox titration of $Q_A$ in the reaction centers from BTAi 1. Actual data points are shown with a theoretical Nernst curve ($E_m = -44$ mV, $n = 1$) superimposed. Note the high potential data points deviate from the normal S-shaped titration curve. This is presumably due to cytochrome $c$ accumulating in the oxidized form, thereby resulting in a disproportionately high population of $P^+$ with respect to lower potential trials.](image-url)
Figure 11. Sequence alignment of the M subunit (A.) and the L subunit (B.). Highlighted areas indicate residues aligned with amino acids within 10Å of C-6 in the Qa ubiquinone of Rb. sphaeroïdes. R. denit- Roseobacter denitrificans; Rb. sphaer – Rhodobacter sphaeroïdes; ORS 278-Bradyrhizobium sp. closely related to BTAi 1; Rp. Viridi- Rhodopseudomonas viridis
“hole” in the redox mediators present. The data suggest that the points in and around -50 mV were not well mediated.

Identification of Amino Acids Constituting the $Q_A$ Binding Pocket

The amino acids of the $Q_A$ binding pocket from the reaction centers of the three species aligned to the binding pocket of $Rb. sphaeroides$ showed no significant differences. Of the differences present, none of the variable amino acids appeared to be potentially significant when viewed to a first approximation using the Swiss Protein Data Bank Viewer©. When the “bubble” was reduced from 10 Å to 7.5 Å, there were no differences between any of the species studied. Figure 11 shows the amino acid sequence alignment of the L and M subunits. There were no unique differences shared by the aerobic photosynthetic bacteria ORS 278 and $R. denitrificans$ compared to the other two species, which perform photosynthetic metabolism when they are anaerobic.

Purification of Reaction Centers from BTAi 1

Many difficulties were encountered when attempting to purify reaction centers from BTAi 1. The method outlined by [Yurkov V., 1998 #42] yielded poor results.

Figure 12. Typical absorption spectrum from the second elution of the DEAE Sepharose column chromatography step.

Typical reaction center spectra were not found when reaction centers were purified following the above mentioned procedure. Figure 12 shows a typical absorption spectrum obtained.
from the second elution of a protein preparation using this procedure.

Typically, the absorption spectrum should show roughly equal extinctions at around 754 nm and 867 nm. The height of the 867 nm band in the figure clearly suggests either there is incomplete dissociation of light-harvesting proteins from the reaction centers or there is a perturbation in the absorption spectrum of the reaction centers in the given conditions (buffer containing 50 mM Tris*HCl, pH = 8.0, 0.1% LDAO) indicating a compromise in the tertiary structure of the polypeptides. Increasing the number of elutions did not improve the spectral quality of the proteins eluted from the column. This was taken as a failure in the method.

Attempts at varying the temperature during the initial solubilization of chromatophores were unsuccessful as well. When the temperature was increased from room temperature to 37°C, the sample turned completely black immediately upon application to the DEAE Sepharose. No further experiments were carried out at temperature higher than room temperature. Repeating the procedure in an environment where everything including the column was kept at 4°C had no positive effect either.

In order to address where the

![Figure 13. Absorption spectrum from reaction centers isolated from BTAi 1. The relative extinction of the 754, 800 and 867 nm bands are identical to that of other purple photosynthetic bacteria, both aerobic and anaerobic.](image)
problem was occurring, light minus dark spectra were taken throughout the procedure. It was found that reaction centers were still fully functioning after solubilization, but not after chromatography.

In light of these problems, experiments were done where LDAO was exchanged for Triton X-100 during the chromatography steps, and Triton X-100 was used in all buffers subsequent to chromatography steps. Also, as a precautionary measure, all steps were done either in the dark or in filtered green light to make sure there was no damaging photochemistry taking place in the reaction centers.

The use of Triton X-100 did yield reaction centers with absorbance spectra that were very similar to those of reaction centers from other species, including both AAP’s and the anaerobic purple bacteria. Figure 13 shows an absorption spectrum from a typical fraction containing reaction centers.

The spectrum shown in figure 13 clearly indicates that photosynthetic reaction centers are the only bacteriochlorophyll-containing proteins in the given fraction. It does not, however, indicate the purity of the reaction center relative to other proteins that might be present. As mentioned earlier, the 280/800 index is unusable because Triton X-100 absorbs light maximally at 280 nm. Other methods such as SDS-PAGE were employed to determine the relative purity of the reaction centers with respect to all proteins that might be present.
Protein aggregation could not be overcome in the gel electrophoresis experiments. Many different denaturation times and temperatures were used. Figure 14 illustrates conditions that result in protein banding patterns indicating aggregation.
The best results appear to have come from denaturation at 100° C for 1 min in 3% SDS (all samples contained 3% SDS), where the aggregates that appear in the high apparent molecular weight region of the gel do resolve. The replacement of β-mercaptoethanol with dithiothreitol had no effect on the banding pattern. A trial where dithiothreitol was added after heating also had no effect, other than to slightly perturb the position of the H subunit band. Protection with iodoacetamide had no visible effect either.

The appearance of a ~55 kD thick band is consistent with the idea that L/L, L/M and M/M dimers could be forming. The higher apparent molecular weight bands could be accounted for by the formation of trimers and tetramers. Since the L and M subunits are highly hydrophobic it is not unreasonable to assume the formation of aggregates that would describe the results in Figure 14. Also, because of the high hydrophobicity of the L and M subunits (polarities = 25.4% and 26.3% for L and M respectively), the apparent molecular weight of the bands should be less than what is predicted by their sequence. This is because membrane-spanning proteins bind up to seven times as much SDS as soluble proteins, and so their charge to mass ratios will be greater. Because of these properties, it is expected that the L and M subunits will migrate farther down a gel than similarly sized water-soluble protein. The theoretical molecular weights of the L and M subunits are 31.5 kD and 34.9 kD respectively (from sequence data in figure 10). The banding pattern observed on the gels is consistent with these theoretical molecular weights.
In order to obtain reaction centers as presented above, the LDAO-dissolved chromatophores had to be eluted through the DEAE Sepharose column twice. The first elution resulted in LHI+RC particles. These particles had roughly the same absorbance spectrum profile as chromatophores (see figure 15), except the 800 nm band is much more prominent, indicating an enrichment of reaction centers compared to light-harvesting proteins.

The profile of the first elution indicated that tail end of the LHI + RC peak was more enriched with reaction centers, which could actually mean that the LHI proteins are not attached to the RC, but rather they elute at roughly the same salt concentration. Figure 16 shows the profile of the first elution.
Figure 16. First elution profile. The LHI + RC particles came off around fraction 13. The salt concentration at the maximal elution of LHI = RC was about 100 mM.

Figure 17. 2nd elution profile. The main band of reaction centers came off at a lower concentration of salt on the second elution (50 mM).
The fractions containing LHI + RC particles were pooled and assayed for activity. The second elution was preceded by the addition of LDAO to 2% in order to ensure complete dissociation between the light harvesting proteins and the reaction centers.

During the second elution, the reaction centers were much more enriched. The absorbance spectra collected from these fractions were consistent with the spectra from other species of photosynthetic bacteria. Figure 17 shows a 2nd elution profile. In the elution profile shown, fractions 17-20 would be pooled and analyzed for activity. Fraction 17 appeared to have the purest reaction centers based on the relative concentration of protein compared to the absorbance at 800 nm.

The method inspired by (Wraight, 1979), which involved the use of LDAO as the only detergent, yielded reasonably pure reaction centers. The procedure currently requires further work, and only qualitative data are available to validate the purification protocol. Reaction centers were found to be inactive upon treatment at 3.0% LDAO. After fractionation with increasing concentrations of ammonium sulfate (in 0.1% LDAO), activity was restored, especially in the second fraction (60% of saturation with ammonium sulfate). Using the purity score described in Materials and Methods, a qualitative description of the isolation and purification procedure can be visualized by Figure 18.
A second elution was attempted on the column of the protein gathered in first elution, but the no viable reaction centers were recovered. This is probably due to the lack of starting material, as little protein was recovered from the first elution. The reaction centers found in ASII and the first protein peak from the first elution were viable, and reasonably pure. Figure 19 shows the absorbance spectrum and the light minus dark spectrum of the protein from the first peak off the column.

Figure 18. Purity score calculated as ∆abs790-810 nm/abs280nm. Dissolved chromat- dissolved chromatophores; ASI-ASIII- ammonium sulfate fractions I-III (35, 60 and 85 % of saturation respectively); elut 1 peak 1 – first colored protein peak off column (first elution); elut 1 peak 2 – second colored protein peak off column (first elution).
The MALDI TOF mass spectroscopy results of the same sample represented in Figure 19 are in agreement with the theoretical molecular weights derived from the sequence data in Figure 11. Unfortunately no sequence data are yet available for the H-subunit, however the molecular weight reported here for BTAi 1 is very representative of

![Absorbance](image1)

Figure 19. Absorbance spectrum and light minus dark spectrum of reaction centers purified from BTAi 1. The spectra represent a fraction from the column following ammonium sulfate precipitation. These spectra compare very well with spectra, published by [Yurkov V., 1998 #42], of other AAP’s as well as with the very well established anaerobic purple bacteria such as *Rhodobacter sphaeroides*. 
other reaction centers studied (Lancaster, 2001). The molecular weights derived from MALDI TOF mass spectroscopy for BTAi 1 are 29.0 kD, H-subunit; 32.4 kD, L-subunit; 34.9 kD, M-subunit. Figure 20 show the MALDI TOF spectrum collected for the protein whose absorbance spectrum is shown in Figure 19.

Figure 20. MALDI TOF mass spectrum of photosynthetic reaction centers from BTAi 1. The top graph represents the actual data from the instrument. The bottom graph is data corrected for polypeptides with a +2 charge instead of +1, which is status quo. The only unexplainable contaminant is the roughly 27 kD peak. The peaks identified agree well with the theoretical molecular weights solved by summing the weights of the amino acids for ORS 278.
Determination of the Stoichiometry of Bactetriochlorophyll:Bacteriopheophytin in BTAi 1 Reaction Centers.

The stoichiometry of bacteriochlorophyll:bacteriopheophytin in BTAi 1 reaction centers was found to be 2.2 ± 0.3. This is taken to mean that there are two bacteriochlorophyll molecules for every one bacteriopheophytin. This is the expected result for a typical reaction center purified from purple photosynthetic bacteria, where the actual distribution of pigments is 4 bacteriochlorophylls and 2 bacteriopheophytins.

Determination of the Presence or Absence of a Bound Cytochrome c to the Reaction Center

Unpublished photooxidation data from Fleischman and Kramer previously suggested that no bound tetraheme cytochrome c subunit was present on the reaction center of BTAi 1. SDS PAGE experiments with whole chromatophores from BTAi 1 and \textit{R. viridis} as a positive control provide more evidence that reaction centers from BTAi 1 do not have a bound cytochrome c subunit. The \textit{R. viridis} trial yielded a bright blue band at 40 kD, which is

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure21.png}
\caption{TMBZ developed gel. Lane 1, 5 µg cytochrome c; Lane 2, 1 µg cytochrome c; Lane 3, \textit{R. viridis} chromatophores; Lane 4 and Lane 5, BTAi 1 chromatophores; Lane 6, molecular weight marker. (Cytochrome c in lanes 1 and 2 is horse heart cytochrome c from Sigma).}
\end{figure}
precisely where the cytochrome c subunit would be expected to be found. No positive bands were found for BTAi 1 in this region. Figure 21 shows the TMBZ-developed gel.

Qualitative Determination of the Chemical Species Occupying the $Q_a$ site in reaction centers from BTAi 1.

All attempts at determining the species of $Q_A$ in BTAi 1 were unsuccessful. It is assumed that not enough starting material was used for the determination. Because of the prevalence of quinones throughout the electron transfer system under study, exceedingly pure preparations of reaction centers would be necessary to definitively assign a particular chemical species to $Q_A$. 

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Discussion

Purification of Reaction Centers

Both purification procedures outlined in the thesis yielded relatively pure reaction centers. For the purposes of developing the procedure for purifying reaction centers from BTAi 1, the method involving ammonium sulfate precipitation and LDAO as the only detergent is preferred so that the relative purity after individual steps in the procedure can be easily and non-destructively assayed. Once the reaction centers are in 0.1% LDAO, there seems to be no inhibition of activity.

The major advantage of the last method (ammonium sulfate precipitation) outlined is the apparently complete removal of light-harvesting proteins before the sample is exposed to chromatography. Regarding the procedures that use chromatography exclusively, it is unclear whether the light-harvesting proteins happen to elute from the column at the same or similar salt concentrations, or if they are actually bound to the reaction center, indicating incomplete solubilization by the detergent. Unpublished data from size-exclusion chromatography indicate that the light-harvesting proteins were bound to the reaction center. This observation suggests that the action of ammonium sulfate precipitating reaction centers also dissociates them for light-harvesting proteins, because the second ammonium sulfate
fraction yielded a fraction with functional reaction centers and little or no light-harvesting protein present.

Another advantage of the final purification procedure outlined deals with the limitations of how much starting material the procedure that uses only chromatography can use. There is a definite limit to how much protein will bind the charged resins on the DEAE Sepharose column, and so initially purifying the reaction centers by ammonium sulfate precipitation would allow for a significant increase in the amount of starting material used. For future studies, including the identification of the QA species, a higher quantity of reaction centers will be helpful if not necessary.

During both of the successful procedures described, it was found that doing the purification in the dark or in green filtered light was necessary to preserve the functionality of the reaction centers. Green light is not absorbed by photosynthetic reaction centers, which absorb blue light (carotenoid) and red to infrared light (bacteriochlorophyll). All data that were reported were collected from preps done in the dark or in green filtered light.

_test Test of Hypothesis: “Is the Midpoint Potential of QA in Reaction Centers from BTAi 1 Higher than those of QA Quinones Found in Anaerobic Photosynthetic Bacteria”_

The beginning hypothesis of this thesis was that something is inherently different about the photosynthetic reaction centers found in BTAi 1, compared to the reaction centers found in their anaerobic counterparts. The hypothesis was based on the observation that QA was overreduced under anaerobic conditions, thereby blocking photosynthetic electron transport. The data collected to test this hypothesis indicate that in fact there is very little difference between the reaction center found in BTAi 1 and
those of *Rb. sphaeroides*. The sequence alignment of the L and M subunits from *Bradyrhizobium* strain ORS 278, which was used because it is very closely related to BTAi 1 (the sequencing work has not been done on BTAi 1), clearly shows very little difference in the binding pocket of QA. Based on the sequence alignment, the likelihood of QA in BTAi 1 having a different midpoint potential versus those of anaerobic photosynthetic bacteria is very low.

Figure 22 shows the QA binding pocket for *Rb. sphaeroides*. None of the amino acids reported in the literature (Wells *et al.*, 2003) to hydrogen bond with QA are absent from the sequences of ORS 278 or *R. denitrificans* (another AAP). The only amino acid that is not the same in every species listed in the sequence alignment is highlighted. The two closest atoms between this amino acid and the ubiquinone molecule are almost 5Å apart, making it very unlikely to affect the immediate environmental dielectric constant for the medium surrounding the quinone. Furthermore, the variation in the R groups between the residues that are different for each of the species is only in size, not chemical property (i.e. they are all hydrophobic).
It is expected that $Q_A$ in BTAi 1 will turn out to be either ubiquinone-n or menaquinone-n. The apparent similarity of reaction centers from BTAi 1 to those of anaerobic photosynthetic bacteria as well as other AAP’s would make it very surprising to find an alternative species for $Q_A$. Both structural and electrochemical differences would be expected for a $Q_A$ if it were anything other than ubiquinone-n or menaquinone-n.

The spectral data collected for both whole chromatophores and for isolated reaction centers indicate that the placement and function of the bacteriochlorophylls present are exactly the same as in the anaerobic photosynthetic bacteria. The stoichiometry of bacteriochlorophyll to bacteriopheophytin is the same in BTAi 1 as in other anaerobic and aerobic photosynthetic bacteria, providing further evidence that the photosynthetic reaction centers are very similar to those of *Rb. sphaeroides* and other photosynthetic bacteria.

The only possibility left for there to be a significant difference in the BTAi 1 reaction center versus those of anaerobic bacteria lies in the H-subunit, which to date has not been sequenced. The H-subunit is thought to be responsible for delivering protons to $Q_B$ (Takahashi & Wraight, 1996). None of the experiments done, with the exception of mass spectroscopy, indicate one way or the other if the H-subunit in BTAi 1 is similar to other reaction centers. The H-subunit molecular weight indicates that it is at least comparable to H-subunits from those of other species.

While the basic machinery for photosynthetic charge separation in BTAi 1 is the same as in the anaerobic photosynthetic bacteria, the basis for photosynthetic metabolism in BTAi 1 might be very different from that in other photosynthetic bacteria. It has been
well documented that AAP’s do not produce bacteriochlorophyll if a good carbon source such as glucose is present (Koblizek et al., 2003). Likewise, BTAi 1 produces bacteriochlorophyll at variable levels with respect to the carbon source available (Evans, Forquer and Fleishman, unpublished observation), and makes the most bacteriochlorophyll when no carbon source other than yeast extract is added to the growth media. Initial O$_2$ uptake for whole bacteria in a variety of carbon sources indicates that the O$_2$ uptake rate correlates somewhat inversely with bacteriochlorophyll production.

It should be made clear that in BTAi 1, and probably other AAP’s, the quinone pool is filling up under anaerobic conditions, and that Q$_A$ is definitely becoming overreduced. At issue is whether or not Q$_A$ is becoming overreduced because there is something inherently different about Q$_A$ electrochemistry. Because it appears as though there is not something different about Q$_A$ in BTAi 1, Q$_A$ is probably overreduced because of the overreduction of the quinone pool, not because of a higher midpoint potential, as originally proposed.

Tichi et al. (Tichi et al., 2001) have shown that anaerobic photosynthetic bacteria are capable of performing reversed electron flow via Complex I (NAD/NADH ubiquinone oxidoreductase). They showed that anaerobic photosynthetic bacteria which had a mutant Complex I could not perform photosynthetic metabolism under anaerobic conditions. Reversed electron flow would be a perfect mechanism to maintain the redox poise of the quinone pool, something the AAP’s are clearly unable to do under anaerobic conditions. The purification of Complex I should be done to see if the basic mechanism of the enzyme in BTAi 1 is consistent with that of other anaerobic photosynthetic
bacteria. Also, sequencing of the genes that make up Complex I would helpful, again to check for, similarities with anaerobic photosynthetic bacteria.

Photosynthetic metabolism in BTAi 1 may act as a supplement to, and not a replacement for oxidative metabolism. Coleman and Fleischman (unpublished data) have shown that the photosynthetic metabolism in BTAi 1 appears to contribute to the total output of nitrogen fixation in the leguminous plant _Aeschynomene indica_. This may be the primary reason for photosynthetic metabolism in BTAi 1, as nitrogen fixation is costly from an energetic standpoint, costing 18 moles of ATP for each mole of N₂ fixed. In addition, Giraud et al. (Giraud et al., 2000) showed the photosynthetic apparatus of _Bradyrhizobium ORS278_ greatly increased the nodulation efficiency on the host plant.

**Comparison of BTAi 1 to Other Anoxygenic Aerobic Phototrophs**

The original inspiration for the stated hypothesis of this thesis was based on research done on other anoxygenic aerobic phototrophs. Vermeglio and coworkers [Yurkov V., 1998 #42] reported midpoint potentials for QA in other AAP’s that could account for an abnormal overreduction of QA. During the experiments done for this thesis research, it was found that a significant (30-50 min) amount of time was required for the system (QA, mediators, and electrode) to reach equilibrium. (Schwarze et al., 2000) reported a midpoint potential of –50 mV for _Roseobacter denitrificans_, another APP. This, along with the very consistent data collected for BTAi 1, suggest that the mechanism of overreduction of QA is not a quinone with a higher midpoint potential, rather it is the lack of redox homeostasis under anaerobic conditions that shut down photosynthetic electron transport in AAP’s.
## Appendix A

### Growth Media for BTAi 1 (Evans’ Media)

Recipe for 4 Liters

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (grams unless noted otherwise)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_2$HPO$_4$</td>
<td>6.68</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>6.00</td>
</tr>
<tr>
<td>MgSO$_4$*7H$_2$O</td>
<td>0.80</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>3.48</td>
</tr>
<tr>
<td>Citric Acid from 0.05 M stock solution</td>
<td>4.00 mL</td>
</tr>
<tr>
<td>FeSO$_4$*7H$_2$O</td>
<td>56 mg</td>
</tr>
<tr>
<td>MoO$_4$*2H$_2$O (as 1.4 mM (NH$_4$)$_6$Mo$_7$O$_2$$_4$)</td>
<td>4.00 mL</td>
</tr>
<tr>
<td>Trace Metals*</td>
<td>0.80 mL</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>2.00</td>
</tr>
<tr>
<td>Concentrated KOH</td>
<td>to pH 6.75</td>
</tr>
</tbody>
</table>

Ingredients should be added in the order shown to promote hasty solvation.


Solution should be autoclaved, and stored under sterile conditions.

If solid media is desired, add 1.5 g/100 mL agar agar from Difco®
Literature Cited:


