Photochemical Electron Transfer in Homodimeric Type I Photosynthetic Reaction Centers

A Dissertation in
Biochemistry, Microbiology and Molecular Biology

by

Bryan H. Ferlez

©2016 Bryan H. Ferlez

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2016
The dissertation of Bryan H. Ferlez was reviewed and approved* by the following:

John H. Golbeck  
Professor of Biochemistry and Biophysics  
Professor of Chemistry  
Dissertation Advisor  
Chair of Committee

Donald A. Bryant  
Ernest C. Pollard Professor in Biotechnology  
Professor of Biochemistry and Molecular Biology

B. Tracy Nixon  
Professor of Biochemistry and Molecular Biology

Carsten Krebs  
Professor of Chemistry  
Professor of Biochemistry and Molecular Biology

Manish Kumar  
Assistant Professor of Chemical Engineering

Scott B. Selleck  
Professor of Biochemistry and Molecular Biology  
Head of the Department of Biochemistry and Molecular Biology

*Signatures are on file in the Graduate School
ABSTRACT

Homodimeric Type I reaction centers (RCs) convert sunlight into chemical potential by a series of energetically favorable photochemical electron transfer (ET) reactions. The product is an oxidized special pair of bacteriochlorophylls (BChls) and a reduced low-potential iron-sulfur (FeS) cluster. The latter reduces soluble ferredoxins that, in turn, act as electron donors to the cellular metabolism. Cytochromes return the RC to the ground state by reducing the oxidized BChl special pair. The work contained in this dissertation expands our understanding of these fundamental ET pathways by quantifying the thermodynamic and kinetic properties of RCs from both anaerobic (Heliobacterium modesticaldum) and aerobic (Chloracidobacterium thermophilum) phototrophs.

In heliobacteria, the midpoint potentials of $A_0$ (-854 mV), $F_X$ (-504 mV), $F_A$ (-452 to -480 mV) and $F_B$ (-524 to -533 mV) are 50-200 mV more oxidizing than the respective cofactors in Photosystem I (PSI). The data suggest that the energy gap between $A_0$ and $F_X$ is poised to maintain free energy for the reduction of ferredoxins while minimizing competition between charge recombination and cytochrome reduction of the oxidized BChl dimer.

In C. thermophilum, the kinetics of both the forward and charge
recombination ET reactions are similar to those of the RCs of the anaerobic phototrophs. In contrast, the PscB protein harboring the $F_A$ and $F_B$ FeS clusters is more tightly bound to the RC than in either of the analogous proteins in the anaerobic species. The strength of this interaction resembles that between PsaC and PSI and may similarly provide oxygen stability to the $F_A$ and $F_B$ clusters.

When heliobacteria are exposed to oxygen, BChl $g$ and BChl $g'$ are oxidized primarily to $8^{1}$-OH Chl $a_F$. Despite losses in energy transfer from the antenna, charge-separation decreases nonlinearly with respect to the amount of oxidized BChl $g$. These data suggest that the HbRC is more robust than originally thought and can function with a heterodimeric special pair (BChl $g'/Chl \, a'_{ox}$).
Chapter 1 Introduction ........................................................................................................... 1
  1.1 Overview of Type I Reaction Center Photochemistry .................................................. 2
  1.2 The Homodimeric Type I RC Core ................................................................................. 5
  1.3 The Bound Electron Transfer Cofactors of Homodimeric Type I Reaction Centers .......................................................... 7
    1.3.1 The special pair P_{800}/P_{840} .......................................................................... 7
    1.3.2 The primary acceptor A_0 ...................................................................................... 9
    1.3.3 The secondary acceptor A_1 ............................................................................... 10
    1.3.4 Interpolypeptide FeS cluster F_X ........................................................................ 13
    1.3.5 Summary ............................................................................................................ 16
  1.4 Electron Transfer Past F_X: The Acceptor Side FeS Clusters F_A and F_B ................. 17
    1.4.1 Helioacteria: PshBI and PshBII .......................................................................... 17
    1.4.2 Green sulfur bacteria: PscB ................................................................................ 20
    1.4.3 Chloracidobacteria: PscC .................................................................................. 22
    1.4.4 Summary ........................................................................................................... 23
  1.5 The Secondary Electron Donor to P_{800}/P_{840}: C-Type Cytochromes .................. 24
    1.5.1 Helioacteria: lipid anchored cytochrome c_{553} ................................................. 25
    1.5.2 Green sulfur bacteria: integral membrane cytochrome PscC .............................. 27
    1.5.3 Chloracidobacterium thermophilum cytochromes ............................................. 29
  1.6 Dissertation Overview ................................................................................................. 30
  1.7 References ................................................................................................................... 32
  1.8 Figures ......................................................................................................................... 43
  1.9 Tables ........................................................................................................................ 45

Chapter 2 Thermodynamics of the Electron Acceptors in Helio bacterium modesticaldum: An Exemplar of an Early Type I Photosynthetic Reaction Center ................................................................................................................................. 47
  2.1 Abstract ....................................................................................................................... 48
  2.2 Introduction ................................................................................................................ 49
  2.3 Materials and Methods ............................................................................................. 54
    2.3.1 Growth of Helio bacterium modesticaldum and preparation of membranes .......... 54
    2.3.2 Isolation of heliobacterial reaction centers ......................................................... 55
    2.3.3 Redox potentiometry of the HbRC by optical spectroscopy ............................... 55
    2.3.4 Ultra-fast transient absorption spectroscopy ...................................................... 56
    2.3.5 EPR spectroscopy on carbonate-washed membranes ....................................... 58
    2.3.6 Redox potentiometry of the HbRC by EPR spectroscopy ................................... 59
Chapter 3 Electron Transfer Processes in Homodimeric Type I Reaction Centers from Heliobacteria, Green Sulfur Bacteria, and Chloracidobacteria 111

3.1 ABSTRACT 112

3.2 INTRODUCTION 113

3.3 MATERIALS AND METHODS 118

3.3.1 Preparation and purification of heliobacterial reaction centers 118

3.3.2 Preparation and purification of chlorosome-depleted membranes from C. tepidum 119

3.3.3 Preparation and purification of chlorosome-depleted membranes from C. thermophilum 120

3.3.4 Time-resolved optical spectroscopy 120

3.3.5 Redox titration of the $P_{860}^{\text{F}_X}$ RP from CabRC 122

3.3.6 Transient and field-modulated time-resolved continuous-wave EPR spectroscopy 124

3.3.7 ODMR spectroscopy 125

3.4 RESULTS 127

3.4.1 Charge recombination $^3P_{860}$ in HbRCs 127

3.4.2 trEPR spectra of HbRCs and GSbRCs under oxidizing and reducing conditions 128

3.4.3 ODMR detection of $^3P_{860}$ 130

3.4.4 Simulation of triplet state polarization as a function of the zero-field splitting parameters $D$ and $E$ 132

3.4.5 trEPR detection of $^3P_{860}$ in C. thermophilum membranes 134

3.4.6 Oxidation-reduction response of the CabRC RP 135

3.4.7 Room temperature charge recombination in CabRCs 136
LIST OF FIGURES

**Figure 1.1**: Overview of homodimeric reaction center complexes and electron transfer components.......................... 43

**Figure 2.1**: Room temperature optical titration of the long-lived $P_{800}^+$ photobleaching signal .......................................................... 92

**Figure 2.2**: Ultra-fast time-resolved optical spectra of oxidized and reduced HbRCs at room temperature................................. 94

**Figure 2.3**: Redox response of the $P_{800}^+$ radical using time-resolved field-modulated CW EPR at 90 K ........................................ 96

**Figure 2.4**: Redox titration of the flash-induced $P_{800}^+$ radical at 90 K ........ 98

**Figure 2.5**: Chemical and light-induced $F_X^-$ difference spectra measured with CW EPR at 4.3 K................................. 100

**Figure 2.6**: Direct redox titration of the $F_X^- S = 3/2$ CW EPR Signal ........ 101

**Figure 2.7**: Redox titration of the $P_{800}^+$/$F_X^-$ transient EPR radical pair at 90 K ................................................................. 103

**Figure 2.8**: Redox titrations of PshBI, PshBII, and HM1_2505 using CW EPR at 15 K ................................................................. 105

**Figure 2.9**: Sequence alignment of PsA, PsB, and PshA ................. 107

**Figure 2.10**: $P_{800}^+$ lifetime modeled as a function of the free energy difference between $A_0$ and $F_X$ ........................................ 108

**Figure 2.11**: Comparison of the thermodynamics and kinetics of the ET chain in Photosystem I and the HbRC ........................................ 109

**Figure 3.1**: Jablonski diagram of the low-temperature ET pathways in the HbRC ................................................................. 149

**Figure 3.2**: $rEPR$ spectra of GSbRCs and HbRCs under oxidizing and reducing conditions ................................................................. 150
Figure 3.3: FDMR spectra of HbRCs under oxidizing and reducing conditions

Figure 3.4: Deconvolution of FDMR spectra of the HbRC

Figure 3.5: Simulation of the dependence of the $^3P_{840}$ and $^3P_{800}$ trEPR spectra on the ZFS parameter $E$

Figure 3.6: Simulation of the $^3P_{840}$ and $^3P_{800}$ trEPR spectra from GSbRCs and HbRCs

Figure 3.7: $^3P_{840}$ formation in chlorosome-depleted membranes of *C. thermophilum*

Figure 3.8: Redox behavior of $P_{840}^+Fx^-$ radical pair signal in chlorosome-depleted *C. thermophilum* membranes

Figure 3.9: $P_{840}^+$ charge recombination kinetics of chlorosome-depleted *C. thermophilum* membranes at 90 K

Figure 3.10: Room temperature optical characterization of chlorosome-depleted *C. thermophilum* membranes at 840 nm

Figure 4.1: Chemical structures of BChl *g*, Chl $a_F$, and 8'-OH Chl $a_F$

Figure 4.2: Optical characterization of the light and oxygen mediated conversion of BChl *g* to Chl $a_{ox}$

Figure 4.3: Optical characterization of the oxygen mediated conversion of BChl *g* to Chl $a_{ox}$ in the absence of light

Figure 4.4: HPLC and mass spectrometry analysis of BChl *g* oxidation products

Figure 4.5: HbRC activity during BChl *g* oxidation

Figure 4.6: Low-temperature charge recombination kinetics during oxidation of BChl *g*
Figure 4.7: Spin-polarized $P^\ast F_X^-$ radical pair at X- and Q-band before and after BChl $g$ oxidation ................................................................. 210

Figure 4.8: Characterization of ISC triplets formed during BChl $g$ oxidation ......................................................................................................................... 211
LIST OF TABLES

**Table 1.1:** Homodimeric RC complex components and electron transfer cofactors .................................................................................................................. 45

**Table 2.1:** EPR simulation parameters for F$_X^-$ ................................................................. 110

**Table 3.1:** trEPR simulation parameters for GSbRCs and HbRCs................................. 162

**Table 3.2:** FDMR derived zero-field splitting (ZFS) parameters D and E for $^3P_{800}$ .................................................................................................................................................... 163

**Table 3.3:** ZFS parameters for $^3P_{840}$ (CabRC), $^3P_{840}$ (GSbRC), and $^3P_{800}$ ...... 164

**Table 4.1:** Duration of the slow kinetic phase during HbRC exposure to O$_2$ ..................................................................................................................................................... 213
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_0$</td>
<td>primary acceptor</td>
</tr>
<tr>
<td>$A_1$</td>
<td>secondary acceptor (phylloquinone in photosystem I)</td>
</tr>
<tr>
<td>Asc</td>
<td>sodium ascorbate</td>
</tr>
<tr>
<td>BChl</td>
<td>bacteriochlorophyll</td>
</tr>
<tr>
<td>CabRC</td>
<td>chloracidobacterial reaction center</td>
</tr>
<tr>
<td>Cbp</td>
<td>carotenoid binding protein</td>
</tr>
<tr>
<td>Chl</td>
<td>chlorophyll</td>
</tr>
<tr>
<td>CW</td>
<td>continuous-wave</td>
</tr>
<tr>
<td>Cyt</td>
<td>cytochrome</td>
</tr>
<tr>
<td>$E_m$</td>
<td>midpoint potential</td>
</tr>
<tr>
<td>EPR</td>
<td>electron paramagnetic resonance</td>
</tr>
<tr>
<td>eV</td>
<td>electron volt</td>
</tr>
<tr>
<td>$F_A$</td>
<td>$[4Fe-4S]^{2+/1+}$ cluster bound by bacterial-type ferredoxins</td>
</tr>
<tr>
<td>$F_B$</td>
<td>$[4Fe-4S]^{2+/1+}$ cluster bound by bacterial-type ferredoxins</td>
</tr>
<tr>
<td>$F_X$</td>
<td>interpolypeptide $[4Fe-4S]^{2+/1+}$ cluster</td>
</tr>
<tr>
<td>Fd</td>
<td>ferredoxin</td>
</tr>
<tr>
<td>FeS</td>
<td>iron-sulfur</td>
</tr>
<tr>
<td>FMO</td>
<td>Fenna-Matthews-Olson protein</td>
</tr>
<tr>
<td>FNR</td>
<td>ferredoxin:NADP$^+$ oxidoreductase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>GSbRC</td>
<td>green sulfur bacterial reaction center</td>
</tr>
<tr>
<td>HbRC</td>
<td>heliobacterial reaction center</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>ISC</td>
<td>intersystem crossing</td>
</tr>
<tr>
<td>NaSCN</td>
<td>sodium thiocyanate</td>
</tr>
<tr>
<td>ODMR</td>
<td>optically detected magnetic resonance</td>
</tr>
<tr>
<td>$P_\lambda$</td>
<td>primary donor, $\lambda$ = wavelength of maximum absorbance</td>
</tr>
<tr>
<td>PMS</td>
<td>1-methoxy-5-methylphenazinium methyl sulfate</td>
</tr>
<tr>
<td>PSI</td>
<td>photosystem I</td>
</tr>
<tr>
<td>PSII</td>
<td>photosystem II</td>
</tr>
<tr>
<td>RC</td>
<td>reaction center</td>
</tr>
<tr>
<td>Redox</td>
<td>reduction-oxidation</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RP</td>
<td>radical pair</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SDT</td>
<td>sodium dithionite</td>
</tr>
<tr>
<td>trEPR</td>
<td>transient electron paramagnetic resonance</td>
</tr>
<tr>
<td>TMBZ</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>half-life</td>
</tr>
<tr>
<td>$\tau$</td>
<td>lifetime</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my advisor and mentor, Dr. John H. Golbeck. I will forever be thankful for the incredible breadth of academic opportunities that you have afforded me. Your professional and scientific example is one that I will strive to emulate throughout my career.

I am also grateful to have had the opportunity to work with, and learn from, each of my distinguished committee members. I would like to thank Dr. Bryant for his guidance throughout my PhD and for the opportunity to work with him and his lab on a number of projects including *C. thermophilum*, Dr. Kumar for his endless professional and academic advice as well as the opportunity to work so closely with his lab, Dr. Krebs for the opportunity to contribute and participate in the Bioinorganic Workshop, and Dr. Nixon for his instruction and help with the single-particle TEM work on the heliobacterial RC. I would also like to extend my sincere thanks to Dr. Art van der Est from Brock University for his continuing support, instruction, and collaboration.

I would also like to thank all of my past and present colleagues in the Golbeck and Bryant labs, specifically Adam Perez, Dr. Marcus Tank, Karim Walters, Dr. Junlei Sun, and Dr. Carolyn Lubner.

Finally, to my wife, Emily, and my daughter, Mae, this PhD simply would not have been possible without you. Thank you for your sacrifice and support.
Chapter 1

Introduction
1.1 Overview of Type I Reaction Center Photochemistry

Photosynthetic reaction centers (RCs) are membrane-bound dimeric protein complexes that function to transform light energy into chemical potential. RCs can be divided into two distinct classes based on the identity of the terminal cofactor in their electron transfer (ET) chain. In Type I RCs this cofactor is an iron-sulfur (FeS) cluster and in Type II RCs it is a quinone. The Type I RCs can be further subdivided as either homodimeric or heterodimeric. Photosystem I (PSI) is the only example of a heterodimeric Type I RC and is found exclusively in oxygenic photosynthetic organisms such as cyanobacteria, plants and algae. Homodimeric Type I RCs are present in three phototrophic bacterial phyla: *Firmicutes* (heliobacteria), *Chlorobi* (green sulfur bacteria), and *Acidobacteria* (chloracidobacteria)\(^1\). The first two of these phototrophs grow under strict anoxic conditions. In contrast, the chloracidobacterial species *thermophilum*, which was isolated from a hot spring microbial mat, experiences temporal cycling between oxic and anoxic conditions throughout the day and can be cultured aerobically in the laboratory\(^2\)-\(^4\).

Light-driven ET in homodimeric Type I RCs is achieved by the participation of two functional domains, the light-harvesting antenna and the ET chain. The light-harvesting antenna is comprised mainly of
(bacterio)chlorophylls ((B)Chls), which enlarge the absorptive crosssectional area and concomitantly increase the likelihood for the absorption of a photon of light. The ET chain consists of a special pair of BChl pigments that serve as the primary electron donor (P) to two equivalent branches of cofactors that vector an electron across the membrane to a terminal FeS cluster, Fx.

The RCs of green sulfur bacteria (GSB) and *C. thermophilum* also associate with extrinsic structures known as chlorosomes that further expand the light harvesting capacity. These structures, which contain hundreds of thousands of additional BChl pigments, are encapsulated in a protein embedded lipid monolayer. In combination with the associated Fenna-Matthews-Olson (FMO) protein, they capture and funnel excitation energy to the RC. The mechanism and arrangement of these complicated antenna complexes is beyond the scope of this dissertation and will not be addressed further.

Upon absorption of a photon by an antenna (B)Chl, the energy is converted to potential energy in the form of a high-energy excited singlet state (*BChl*). This excited state migrates throughout the antenna until it reaches P. The induced electronic transition from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital
(LUMO) produces the excited singlet state \(1^*P\). Direct ET to a proximal primary acceptor \((A_0)\) quenches this low-potential excited state in 25 picoseconds (ps), generating the first charge-separated state \(P^*A_0^-\). \(A_0^-\) is then reoxidized by forward ET to \(F_X\) in 500-800 ps. This extends the lifetime of the charge-separated state by increasing the distance between the donor and acceptor pairs \((P^*F_X^-)\). If the forward ET pathway to \(F_X\) is blocked, the lifetime of \(P^*A_0^-\) lasts only a few ten's of nanoseconds before the energy is dissipated by charge recombination between \(A_0^-\) and \(P^*\). This leads to the regeneration of the ground state via the triplet state of the primary donor \((^3P)\), \(P^*A_0^- \rightarrow ^3PA_0 \rightarrow PA_0\).

The lifetime of the \(P^*F_X^-\) charge-separated state can be increased even further if an extrinsic electron donor such as a cytochrome (Cyt) is available to reduce \(P^*\) prior to charge recombination with \(F_X^-\) \((\text{Cyt}_{\text{red}}P^*A_0F_X^- \rightarrow \text{Cyt}_{\text{ox}}PA_0F_X^-)\). This extrinsic ET step traps the RC in a state incapable of unproductive charge recombination, thereby allowing more time for soluble electron carriers to be reduced by the RC. Similarly, the acceptor side of the RC can be extended past \(F_X\) by extrinsic electron acceptors. In Type I RCs, bacterial-type ferredoxins that bind two \([4\text{Fe}-4\text{S}]^{2+/-1+}\) clusters (\(F_A\) and \(F_B\)) act either as mobile electron acceptors or as fixed ET subunits that subsequently reduce soluble ferredoxins.
1.2 The Homodimeric Type I RC Core

The pshA gene in heliobacteria and the pscA genes in GSB and chloracidobacteria code for proteins that contain 11 predicted transmembrane α-helices. The resulting homodimeric protein cores (PshA)\textsubscript{2} and (PscA)\textsubscript{2} bind the ET cofactors (see section 1.3)(Table 1.1), the (B)Chl antenna molecules, as well as minor amounts of carotenoid and quinone pigments\textsuperscript{3,21-26}.

The BChl of both the GSB and chloracidobacterial antenna bound by the homodimeric Type I RC core is Mg-BChl \textit{a}\textsuperscript{26,27}. These RCs also contain two molar equivalents of either Mg-BChl \textit{a}’ in GSB or Zn-BChl \textit{a}’ in \textit{C. thermophilum}, per RC (where the prime designation refers to the alternate stereochemical configuration of the hydrogen and carboxymethyl groups on the asymmetric 13\textsuperscript{2} carbon atom of ring E)\textsuperscript{26, 28, 29}. Based on a 2:1 stoichiometry with respect to the RC core the Zn-BChl \textit{a}’ and Mg-BChl \textit{a}’ pigments are predicted to play in a role in ET rather than in light-harvesting (see section 1.3.1)\textsuperscript{26, 28, 29}. In contrast, heliobacterial RCs (HbRCs) contain almost exclusively BChl \textit{g}, which shares structural similarity to the tetrapyrrole rings of both Chl \textit{a} (C-3 vinyl group, ring A) and BChl \textit{b} (C-8 ethylidene group, ring B)\textsuperscript{30, 31}. The exocyclic C-8 ethylidene group is responsible for the epimerization and oxygen liability of BChl \textit{g} and yields
Chl $a_{F(ox)}$ upon exposure to light and oxygen (where F represents an esterifying farnesol and (ox) represents oxidized products of BCHl $g$ that are spectroscopically equivalent to Chl $a_F$)$^{32-35}$. Similar to the RCs of GSB (GSbRC) and chloracidobacteria (CabRC), HbRCs contain two molar equivalents of the C-13$^2$ epimer of the predominant BCHl (BChl $g'$) that is considered to constitute the special pair (see section 1.3.1)$^{31}$.

All three representative RCs also contain derivatives of Chl $a$. GSbRCs and CabRCs bind Chl $a_{\Delta 2,6P}$ (where $\Delta 2,6P$ signifies the esterifying $\Delta 2,6$-phytadienol tail) in a ratio of 4 and 8 per RC respectively$^{26, 28, 29}$. HbRCs contain 8$^1$-OH Chl $a_F$ in a ratio of two per RC$^{25, 36}$. These Chl $a$ pigments have been shown in the case of the HbRC and GSbRC or are predicted in the case of CabRC to serve as the primary electron acceptor (see section 1.3.2). Additionally, they may also function as antenna pigments in the GSbRC and CabRC since they are present at molar ratios greater than two per RC homodimer$^{12, 13, 26, 28}$.

Homodimeric RCs also bind a small number of carotenoids and quinones. The role of a quinone as an intermediate acceptor is still widely debated and will be discussed below (see section 1.3.3). The carotenoid content is approximately 1-2 molecules per RC in both GSbRCs and HbRCs. In heliobacteria the carotenoid has been identified as 4,4$'$-diaponeurosporene,
whereas in GSB different combinations of rhodopin, chlorobactene and γ-carotene have been detected depending on the individual species\textsuperscript{25, 27, 36}. CabRCs contain a carotenoid with an identical retention time and absorption spectrum to a lycopene standard identified via reverse-phase high-performance liquid chromatography (HPLC). The molar stoichiometry per RC is difficult to determine due to the fact that the RC co-purifies with a unique carotenoid binding protein (Cbp). This extrinsic Cbp binds 6-7 echinenone and deoxyflexixanthin carotenoids per monomer and it associates with the RC in a ratio of ca. 12 Cbp per RC\textsuperscript{26}. Regardless of their identities, these carotenoids carry out two functions. They 1) expand the size and wavelength range of the antenna and/or 2) provide photoprotection by quenching unused excited (B)Chl triplet states in the antenna and \textsuperscript{1}O\textsubscript{2} in solution\textsuperscript{37}. The latter may be important for \textit{C. thermophilum}, which experiences periods of oxic conditions due to the water oxidation activity of the associated cyanobacterial species in the microbial mat\textsuperscript{2, 3}.

\subsection*{1.3 The Bound Electron Transfer Cofactors of Homodimeric Type I Reaction Centers}

\subsubsection*{1.3.1 The special pair \textit{P}_{800}/\textit{P}_{840}}

Two C-13\textsuperscript{2} epimers of BChl \textit{g}, Mg-BChl \textit{a}, and Zn-BChl \textit{a} have been isolated per RC from heliobacteria, GSB and \textit{C. thermophilum}, respectively\textsuperscript{25}. 


27. These pigments have been assigned as P, the special pairs of BChl that serve as the primary electron donor to the ET chain. This assessment is based on analogy with PSI, in which the primary donor P700 is composed of a heterodimeric pair of a Chl a molecule and a C-132 epimer Chl a’ molecule20. In contrast with PSI, the molar ratio of the C-132 epimer in homodimeric RCs indicates that the special pair BChl pigments are homodimers instead of heterodimers: BChl g’/BChl g’, Mg-BChl a’/Mg-BChl a’ and Zn-BChl a’/Zn-BChl a’. All three homodimeric primary donor species can be characterized optically by their bleaching following oxidation. In the HbRC, the wavelength of maximal bleaching is 798-800 nm; in GSbRC it is 830 nm with a shoulder at 840 nm; and in CabRC it is 840 nm12,26,38-40. Therefore, the primary donor BChls are also referred to by an alternate shorthand designation: P798/P800 in HbRCs, P840 in GSbRCs, and P840 in CabRCs.

There are two important functional consequences of the dimeric nature of the primary donor. The first is that the wavelength of maximal bleaching occurs at longer wavelengths (representing a lower energy gap between the ground and singlet excited state) than for the respective monomeric BChl species due to excitonic coupling between the two pigments41. This creates an energetic minimum that functions to ‘trap’ the excitation energy at the site where quenching of *P can occur via rapid ET to
A₀. The second is that the midpoint potential of the P/P⁺ couple is lowered as a result of delocalization of the P⁺ cation radical across two BChls⁴²,⁴³. This effect can be observed by examining the substantially more negative midpoint potentials (Eₘ) and narrower radical line-widths (ΔH) of the dimeric forms of the Type I (B)Chl donors with respect to their monomeric counterparts (e.g. Eₘ[BChlₐ/BChlₐ⁺] = 660 mV and ΔH₉₈₄ₒ⁺ = 1.3 mT v. P₉₄₀GSB = +240-250 and ΔH₉₈₄ₒ⁺ = 0.9 mT; Eₘ[Chlₐ/Chlₐ+] = +740-930 mV and ΔH₉₈₄ₒ⁺ = 0.9 mT v. P₇₀₀ = +490 mV and ΔH₉₇₀ₒ⁺ = 0.7 mT)⁴³,⁴⁴-⁴⁶.

Similar to the triplet states (S = 1) of monomeric (B)Chl (₃(B)Chl) formed by intersystem crossing (ISC) in the antenna, ³P can sensitize molecular oxygen, generating unwanted reactive oxygen species (ROS) such as ¹O₂. Therefore, ³P plays no role in productive photochemistry and instead only results when ET is disrupted such that charge recombination between A₀⁻ and P⁺ occurs. However, because ³P is paramagnetic and is easily detected by specialized electron paramagnetic resonance (EPR) techniques such as transient EPR (trEPR), it serves as an excellent reporter on ET beyond A₀⁴⁷. It should be noted that trEPR can also distinguish between ISC triplets from the antenna bed and P⁺ A₀⁻ recombination triplets (³P), even if they consist of chemically identical pigments.

1.3.2 The primary acceptor A₀
The primary electron acceptors in the HbRC and GsbRC are $8^1$-OH Chl $a_F$ and Chl $a_{\Delta 2,6\beta}$ respectively\textsuperscript{25,28,29,36}. $A_0$ is reduced in 25 ps in the HbRC and GsbRC\textsuperscript{9,10,48}. Subsequent oxidation of $A_0$ typically reflects the forward ET to the secondary electron acceptor in unperturbed RCs. In the HbRC and GsbRC this lifetime has been measured to be between 500-800 ps, which is roughly 1-2 orders of magnitude slower than in PSI\textsuperscript{12,13,49-51}. If the ET chain is blocked or pre-reduced, the kinetics of $A_0^-$ oxidation reflect charge recombination with $P^*$, which has a lifetime of 17 ns in HbRCs and 25 ns in GsbRCs\textsuperscript{15,52,53}.

1.3.3 The secondary acceptor $A_1$

If the ET cofactors in homodimeric RCs are identical to those in PSI, we would expect that a quinone ($A_1$) would serve as the immediate electron acceptor to $A_0$\textsuperscript{20}. The functional association of quinone molecules with the homodimeric RC is supported by the identification of menaquinone (although often in variable, substoichiometric quantities) in RC preparations from all three phyla\textsuperscript{25-27,54,55}. It was initially suggested that the similarity in amino acid sequence of the $A_1$ binding site between PSI, HbRC, and GsbRC was consistent with a quinone-type ET acceptor\textsuperscript{56}. However, following refinement of the PSI structure to 2.5 Å, it was observed that Trp697 on PsaA and Trp677 on PsaB are π-stacked with the two $A_1$ phylloquinones. These
Trp residues are replaced with Arg residues in the amino acid sequence of PshA of heliobacteria and PscA of GSB\textsuperscript{20, 57}.

The absence of the conserved Trp residue and lack of consistent stoichiometric association of menaquinone with any of the purified homodimeric RCs does not preclude the presence of menaquinone in the ET chain. Instead it suggests that the physical and functional properties are different than in PSI. Spectroscopic analyses have so far failed to clarify these differences and models exist that both include and exclude a menaquinone in the ET chain.

The strongest argument against the role of a menaquinone as a functional secondary acceptor in HbRCs is the unaltered ET kinetics of HbRCs following quinone extraction. The lifetime of P\textsuperscript{+} at room temperature before and after quinone extraction was \(\tau_1 = 10\text{–}30\text{ ms}\) in the presence of 10 mM sodium ascorbate and \(\tau_2 = 17\text{ ns}\) in the presence of 10 mM sodium ascorbate and 15 mM sodium dithionite at pH 9.5. The slower 10-30 ms decay was attributed to charge recombination between \(P_{800}^+F_X^-\) (see section 1.3.4) and the faster 17 ns decay to charge recombination between \(P_{800}^+A_0^-\). Similar results have been obtained with GSbRC preparations in which the menaquinone content was below detection. In these samples, ET proceeded to \(F_X\) at temperatures less than 200 K (\(\tau = 50\text{ ms}\)) and at least as far as the
FeS clusters in PscB at room temperature ($\tau_1 \approx 150$ ms, $\tau_2 \approx 2$ s)\textsuperscript{27,58}. Further evidence against the role of menaquinone in forward ET comes from trEPR experiments. In both HbRCs and GSbRCs, the P* contribution of a spin-polarized radical pair is observed to exhibit a net absorptive polarization and contains no contribution from a semiquinone radical. Furthermore, a theoretical treatment of sequential radical pairs suggests that the net polarization results from a short-lived radical pair precursor with a lifetime of 600 to 700 ps\textsuperscript{11}. This analysis is consistent with the optically measured lifetime of $A_0^-$ oxidation of 500 to 800 ps (see section 1.3.3) and suggests that either ET proceeds directly to F$_X$ or indirectly via a cofactor other than a quinone\textsuperscript{33,34}.

In contrast, other data suggests the photoreduction of a menaquinone in forward ET. These experiments typically rely on photoaccumulation procedures, wherein purified RCs are illuminated in the presence of a fast, electron donor during freezing. EPR signals consistent with a semiquinone radical have been observed using these techniques in both purified HbRCs and GSbRCs\textsuperscript{54,59-61}.

These experimental discrepancies suggest that the role of the menaquinone in homodimeric RCs is different than phylloquinone in PSI. Whether this generalization stems from a substantially different distance,
free energy and/or ET rate landscape that support multiple ET pathways such as \((A_0 \rightarrow F_X)\) or \((A_0 \rightarrow F_X \rightarrow A_1)\) or similar pathways with unusual kinetics, as would be the case for \(k_{\text{et}} (A_0^- \rightarrow A_1^-) \ll k_{\text{et}} (A_1^- \rightarrow F_X)\), remains uncertain.

1.3.4 Interpolypeptide FeS cluster \(F_X\)

In PSI, two residues from PsaA and two Cys from PsaB ligate the four iron atoms of the interpolypeptide \([4Fe-4S]^{2+/1+}\) cluster. This cluster, named \(F_X\), serves as the point of convergence for the two branches of ET cofactors. A conserved amino acid sequence containing the two coordinating Cys ligands (FP\(\text{C}x\text{GPxxGGTC}\)) has been identified in all homodimeric RC polypeptides (PshA, Psc\(_{\text{GSB}}\) and Psc\(_{\text{Cab}}\))\(^{62}\). Furthermore, biochemical and spectroscopic evidence fully support the presence of a \([4Fe-4S]^{2+/1+}\) cluster in HbRCs and GSbRCs analogous to \(F_X\) in PSI.

In illuminated and/or chemically reduced HbRCs and GSbRCs, EPR signals distinct from that of the \(F_A\) and \(F_B\) clusters bound by PshBI/PshBII or PscB (see section 1.5) have been assigned to the reduced \([4Fe-4S]^{1+}\) cluster \(F_X^-\). In HbRCs this cluster has a ground spin state of \(S = 3/2\) with major EPR resonances at \(g \approx 5.4\) and \(4.4\)\(^{63}\). In GSbRCs, \(F_X\) can exist in both an \(S = 3/2\) state with major resonances at \(g \approx 5.4\) and \(4.4\) as well as an \(S = 1/2\) state with a broad resonance at \(g \approx 1.77\)\(^{64}\). In HbRCs, the appearance of these EPR
resonances following chemical reduction is concomitant with the loss of the long-lived 15 ms lifetime of $P_{800}^+$ measured optically at room temperature, consistent with its assignment to $P_{800}^+F_X^-$ charge recombination (note the $\sim 17$ ns lifetime due to $A_0^-$ recombination with $P_{800}^+$ was not observed because the bandwidth of the spectrometer limited the first detection point to 10 $\mu$sec$^6$. In GSbRCs, the tight association between the c-type cytochrome PscC and the RC (see section 1.5.2) interfere with the characterization of the charge recombination kinetics of $P_{840}^+F_X^-$ following a single-turnover flash due to rapid ($< 100$ $\mu$sec) reduction of $P_{840}^+$ (see section 1.5.2). However, multi-flash experiments tracking the simultaneous reduction and oxidation of FeS clusters and $P_{840}$ suggested that three FeS clusters are present per RC$^{65}$. These reduced FeS clusters undergo charge recombination with $P_{840}^+$ with lifetimes of 140 ms, 70 ms, and 17 ms following the first, second and third flash. Based on analogy with PSI, these lifetimes were assigned to the charge recombination between $P_{840}^+F_B^-$ ($\tau = 140$ ms), $P_{840}^+F_A^-$ ($\tau = 70$ ms), and $P_{840}^+F_X^-$ ($\tau = 17$ ms)$^{65,66}$.

The charge recombination of $P_{800}^+/P_{840}^+$ and $F_X^-$ in the HbRC and GSbRC changes as the temperature is lowered. In HbRCs the lifetime abruptly switches from 15 ms at room temperature to 2-5 ms at temperatures below 150 K$^{11,53,67}$. In contrast, $P_{840}^+F_X^-$ charge recombination in GSbRCs slows
from 17 ms at room temperature to 30-50 ms at temperatures below 200 K.\textsuperscript{11, 58, 65} Because charge recombination at cryogenic temperatures is measured indirectly by tracking either the loss of the P\textsuperscript{+} radical by EPR or the loss of the optical bleaching of P\textsuperscript{+} by optical spectroscopy, it is possible that the unusual acceleration of the P\textsubscript{800} lifetime in HbRCs at temperatures less than 150 K is due to charge recombination with an intermediate electron acceptor, and not F\textsubscript{X}. The possibility that this kinetic event represents charge recombination from a semiquinone is unlikely because identical kinetic behavior was observed in menaquinone-depleted membranes of \textit{H. chlorum}\textsuperscript{53}. Alternatively, this faster rate may reflect P\textsubscript{800}F\textsubscript{X} charge recombination following a structural perturbation of the RC below the glass transition temperature. Regardless of the chemical identity of the species involved in this accelerated charge recombination, it is not clear why it has not been observed in GSbRCs.

Finally, only indirect estimates exist for the midpoint potential of F\textsubscript{X}. In heliobacteria, the highest estimation comes from indirect titrations of P\textsubscript{800} in membranes and purified RCs from \textit{H. gestii}, which yielded values of \textasciitilde444 mV (n = 1) and \textasciitilde414 mV (n = 2), respectively.\textsuperscript{68} In contrast, a substantially more reducing value was obtained by Nuijs et al. (also using indirect observations of the oxidation state of P\textsubscript{800} and A\textsubscript{0}\textsuperscript{+}) who estimated that the
potential of Fₓ was lower than −560 mV in membranes of *H. chlorum* (no *n* value reported)¹². In GSB, indirect titrations of P₈₄₀⁺ at room temperature are not feasible due to rapid ET from PscC. Nevertheless, application of a quasi-equilibrium model of charge recombination would predict the free energy difference between Fₓ and the lowest potential FeS cluster bound by PscB is close to zero⁶⁵. Based on the experimentally measured midpoint potential of F₈ in PscB (see section 1.4.2), this suggests the midpoint potential of Fₓ may be ~ −501 mV⁶⁹.

1.3.5 Summary

Optical and magnetic spectroscopies have clarified a number of issues related to the primary photochemistry of homodimeric RCs. For example, in the anaerobic GSB and heliobacterial species, charge-separation and secondary ET appear highly similar and do not require a quinone molecule for reduction of Fₓ. In both species, charge recombination between P⁺A₀⁻ and P⁺Fₓ⁻ occur in ~20 ns and ~15 ms, respectively. In contrast, nothing is known about the ET rates in the oxygen-tolerant RC from *C. thermophilum*. Moreover, the thermodynamics of the ET cofactors are poorly understood. For example, the only experimentally measured midpoint potentials for a secondary acceptor are indirect, span ~200 mV, and are variously fit as one- or two-electron processes.
1.4 Electron Transfer Past $F_X$: The Acceptor Side FeS Clusters $F_A$ and $F_B$

1.4.1 Heliobacteria: $PshB\text{I}$ and $PshB\text{II}$

In contrast to PSI, the HbRC interacts with more than one $F_A$ and $F_B$-containing protein$^{70-72}$. The genes that encode these ferredoxins in $H.\ modesticaldum$, $HM1\_1462$ (PshBI) and $HM1\_1461$ (PshBII), constitute a dicistronic operon and are transcribed as a single transcript$^{72}$. PshBI and PshBII are highly similar (63% sequence identity), small bacterial-type ferredoxins that have slightly shorter amino acid sequences than PsaC, the analogous $F_A$ and $F_B$ containing ferredoxin subunit of PSI (54 versus 81 amino acids). PsaC contains a two amino acid insertion at the N-terminus, an eight amino acid insertion between the two CxxCxxCxxxCFeS cluster binding motifs, and a 15 amino acid C-terminal extension that are absent in PshBI and PshBII. The eight amino acid insertion between the two FeS cluster binding motifs has been identified as important for ferredoxin (plant-type, [2Fe2S] cluster) and flavodoxin interactions with PSI, whereas the two additional N-terminal amino acids, in concert with the extended C-terminal domain, are responsible for orienting PsaC onto the PsaA/PsaB PSI RC core through a network of salt bridges and hydrogen bonds$^{73-75}$. In addition, ten ionic bonds between Lys and Arg residues of PsaC (Lys 51, Arg 52 and Arg
and two symmetric Asp residues on PsaA and PsaB form a strong binding interface between PsaC and the PSI core that can only be disrupted using molar concentrations of chaotropic agents such as urea\textsuperscript{74, 76}. The heliobacterial ferredoxins exhibit substantially weaker binding, as shown by their dissociation from the HbRC under relatively low ionic strengths (~100 mM NaCl)\textsuperscript{70}. Therefore, it has been suggested that PshBI and PshBII are themselves mobile electron shuttles, which transiently dock onto the HbRC core and accept one or two electrons before dissociating\textsuperscript{71, 72}. Support for this hypothesis comes from \textit{in vitro} flavodoxin reduction assays in which the light-induced reduction of cyanobacterial flavodoxin is monitored optically in the presence of detergent solubilized HbRCs and sacrificial electron donors\textsuperscript{18, 77}. Unlike F\textsubscript{X}-PSI cores that have been stripped of PsaC, PsaD, and PsaE and retain no ability to support flavodoxin reduction, HbRCs devoid of PshBI or PshBII are able to donate electrons to flavodoxin\textsuperscript{18, 75, 77}. Moreover when PshBI and PshBII are included as electron acceptors, the effect on the kinetics of flavodoxin reduction are consistent with their action as mixed inhibitors\textsuperscript{18}. 

The functional differences between PshBI/PshBII and PsaC can be rationalized by considering the evolutionary pressure associated with the development of oxygenic photosynthesis. As oxygenation of the atmosphere proceeded as a result of the water oxidation activity of photosystem II (PSII),
the value of soluble bacterial-type ferredoxins as electron acceptors was compromised due to the oxygen sensitivity of the FeS clusters. In response to this oxidative pressure PSI and PsaC developed a specific, strong binding interface that rendered the FeS clusters of PsaC resistant to O₂. In contrast, heliobacteria live in an anoxic environment and contain only a Type I RC. The pressure to protect the acceptor side FeS clusters from O₂ is limited to minimal environmental exposure and as a consequence the FeS clusters in PshBI and PshBII are labile to O₂.

If the transient docking model of PshBI and PshBII with the HbRC is correct, the possibility exists that other electron acceptors function similarly. The genome of *H. modesticaldum* contains two additional genes that are predicted to encode low molecular weight bacterial-type ferredoxins capable of binding two [4Fe-4S]²+/1⁺ clusters: *HM1_2505* and *HM1_0860*. *HM1_0860* is located in the Nif operon that encodes the protein machinery for nitrogen fixation and likely serves as the immediate electron donor to the Fe protein which, in turn, supplies electrons for the reduction of N₂ and H⁺ to NH₃ and H₂ by the MoFe protein. Little is known about the fourth bacterial type ferredoxin (*HM1_2505*) except that it is predicted to be only slightly longer in amino acid sequence than PshBI and PshBII (60 v. 54 amino acids...
respectively). Its role in phototrophic metabolism awaits biochemical elucidation.

Although PshBI and PshBII have been shown to accept electrons from the HbRC, the redox potentials of the two [4Fe-4S]^{2+/1+} clusters have yet to be determined\textsuperscript{71, 72}. Initial reports from Heinnickel et al. suggested that the midpoint potential of the two FeS clusters of PshBI from \textit{H. modesticaldum} were different based on the changes observed in the EPR resonances as the solution potential was lowered. At higher solution potentials (dithionite at pH 7.0) a rhombic set of EPR resonances were observed consistent with the selective reduction of only one of the FeS clusters. As the potential was lowered by addition of dithionite at pH 10.0, these resonances were replaced with a new set of resonances that were different than a simple admixture of two separate reduced FeS clusters, suggesting simultaneous reduction of both FeS clusters in the same protein\textsuperscript{70}. This finding was consistent with previous EPR results on membranes of \textit{H. chlorum} that also suggested different midpoint potentials for the F\textsubscript{A} and F\textsubscript{B} clusters\textsuperscript{81}.

\textit{1.4.2 Green sulfur bacteria: PscB}

PscB from \textit{C. tepidum} contains 231 amino acids, including extended N- and C-terminal domains that are lacking in PshBI, PshBII, and PsaC. The extended N-terminal domain contains a number of PxxP motifs that may
form the binding site for complementary SH3 domains, although no biochemical data exist to support this possibility. In addition, the second FeS cluster binding motif contains an additional two amino acids between the third and fourth Cys residues, a deviation from the traditional CxxCxxCxxxC sequence found in PshBl, PshBII, and PsaC. The specific roles of these two extra amino acids as well as the roles of the extended N- and C-terminal domains in binding to the RC and/or extrinsic proteins are unknown.

GSB also contain additional bacterial-type ferredoxins that, in combination with PscD, have been shown to mediate light-driven electron flux between the GSbRC and ferredoxin:NADP+ reductase (FNR) in vitro. This strongly implicates them as mobile electron shuttles between the $F_A$ and $F_B$ clusters of PscB and the ferredoxin-dependent enzymes of cellular metabolism (e.g. pyruvate synthase and 2-oxoglutarate synthase of the reverse tricarboxylic acid (rTCA) cycle and/or FNR). The role of PscB as a bound intermediary ET cofactor between $F_X$ and soluble ferredoxins is supported by the strength of PscB/GSbRC interaction, which can only be disrupted at moderate ionic strengths of 500 mM NaCl. Despite this interaction being stronger than that between PshBl and the HbRC, the FeS clusters $F_A$ and $F_B$ are still sensitive to degradation by O2.
Spectroscopic and biochemical analyses of PscB have been hindered by the fact that unlike PsaC, PshBI, and PshBII, heterologously expressed forms have not yielded to chemical reconstitution using iron and sulfide\textsuperscript{64}. Instead, the redox potentials of the F\textsubscript{A} and F\textsubscript{B} clusters in a native PscB containing GSbRC have been measured \textit{in situ}. The redox potentials, measured by EPR spectroscopy, were 70-80 mV more positive (-441 mV and -501 mV (v. SHE)) than the F\textsubscript{A} and F\textsubscript{B} clusters in PsaC (-530 and -580 mV respectively)\textsuperscript{69,85}. In the absence of additional thermodynamic information on the ET chain, however, the significance of the positive shift in midpoint potentials remains unclear.

\textit{1.4.3 Chloracidobacteria: PscB}

Similar to GSB, the \textit{pscB} gene in \textit{C. thermophilum} is located in an operon containing the genes for the PscA and FMO proteins, and encodes a significantly longer (173 amino acids) bacterial-type ferredoxin than PshBI, PshBII, and PsaC\textsuperscript{3,8}. Furthermore, the second FeS cluster binding motif also contains two extra residues between the third and fourth Cys\textsuperscript{62}. Although RCs devoid of PscB have been purified from \textit{C. thermophilum} following treatment with 2 M sodium thiocyanate (NaSCN), spectroscopic characterization of the bound FeS clusters await characterization\textsuperscript{26}.
If PscB in \textit{C. thermophilum} is bound to the RC in a manner similar to GSB, soluble bacterial-type ferredoxins are expected to couple light-driven ET in the RC to the formation of a proton gradient for cyclic ET (non-cyclic electron flux is unlikely in \textit{C. thermophilum} due to the lack of genes needed for autotrophic carbon fixation\textsuperscript{8}). No genes encoding putative two [4Fe-4S]\textsuperscript{2+/1+} bacterial-type ferredoxins other than \textit{pscB} are annotated in the genome of \textit{C. thermophilum}\textsuperscript{8}. However, \textit{C. thermophilum} does contain two genes encoding [2Fe-2S] cluster plant-type ferredoxins (\textit{Cabther_A1723} and \textit{Cabther_A0979}) similar to those associated with oxygenic photosynthesis. One of these genes, \textit{Cabther_A0979}, is located in the same putative operon as a hypothetical FNR enzyme, \textit{Cabther_A0980}\textsuperscript{8}. A substitution of the O\textsubscript{2} sensitive bacterial-type ferredoxins used by the strictly anaerobic GSB and heliobacteria species with oxygen-tolerant plant-type ferredoxins likely represents an adaptation to oxidizing conditions. This is consistent with the ability to culture \textit{C. thermophilum} aerobically in the laboratory\textsuperscript{4}. Whether or not these putative [2Fe-2S] cluster ferredoxins function in the light driven metabolism of \textit{C. thermophilum} needs to be confirmed biochemically.

1.4.4 Summary

Heliobacteria and GSB possess bacterial-type ferredoxins similar to PsaC in PSI that accept electrons from the RC\textsuperscript{62, 86-88}. Unlike PsaC, these
ferredoxins can be dissociated from the RC using NaCl and their two [4Fe-4S]$^{2+/1+}$ clusters, $F_A$ and $F_B$, are sensitive to $O_2$ degradation. The binding strength and $O_2$ sensitivity of the analogous ferredoxin in RCs from the aerobe $C.\ thermophilum$ are unknown. Although the midpoint potentials of $F_A$ and $F_B$ have been reported for the GSbRC to be $-441$ and $-501$ mV, no quantification of the midpoint potentials of the analogous clusters in heliobacteria or chloracidobacteria exists.

1.5 The Secondary Electron Donor to $P_{800}/P_{840}$: c-type Cytochromes

Homodimeric RCs drive phototrophic metabolism either by cyclic (heliobacteria, chloracidobacteria, and potentially GSB) or non-cyclic ET (GSB). In cyclic ET, ferredoxins reduced by the RC are used to generate a proton motive force and ultimately reduce cytochrome. The proton motive force drives ATP synthesis via ATPase. The cytochrome reduces the photooxidized primary donor and returns the RC to the ground state. In non-cyclic ET, ferredoxins reduced by the RC are reductants for processes such as carbon fixation. In this case, cytochromes still reduce the oxidized primary donor, however the electrons are obtained via oxidation of a variety of organic and inorganic compounds$^7$. Regardless of the role light-driven ET plays in phototrophic metabolism, c-type cytochromes have been confirmed
or predicted to be the immediate electron donors to P\textsuperscript* for all Type I homodimeric RCs. The kinetics and thermodynamics of this extrinsic ET step are critical to both stabilizing the light-induced charge-separated state and supporting phototrophic growth.

1.5.1 *Heliobacteria: lipid anchored cytochrome c\textsubscript{553}*

The first evidence that a cytochrome was linked to reduction of the special pair of BChl g' pigments (P\textsubscript{800}) came from a bleaching of the absorbance of whole cells of *H. chlorum* at 553 nm, a wavelength characteristic of cytochrome c oxidation, following a flash of actinic light\textsuperscript{89}. The decay was observed to be biphasic with half-times of 100 μsec and 700 μsec. An identical absorbance increase was observed simultaneously at 800 nm suggesting direct ET from the cytochrome (Cyt c\textsubscript{553}) to P\textsubscript{800}\textsuperscript{*89}. Biochemical analyses by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed five distinct polypeptides with molecular weights of approximately 14, 16, 18, 30 and 50 kDa that stained positive by the heme reactive compound 3,3',5,5'-tetramethylbenzidine (TMBZ)\textsuperscript{90}. The most abundant of these cytochromes was later identified to be the 16 kDa cytochrome encoded by the petJ gene\textsuperscript{90}. PetJ is posttranslationally modified by a covalent thioether bond between Cys 23 and a diacylglycerol molecule containing two fatty acids (palmitate and stearate) attached via
methyl ester linakges. The Cys 23 residue harboring this membrane-anchoring modification functions as the N-terminal +1 residue after the preceding 22 amino acid leader sequence is cleaved. The midpoint potential of this monoheme cytochrome was measured in native and heterologously expressed proteins, as well as in native membranes of *H. chlorum*, to be between +170 mV and +215 mV (v. SHE). This result is consistent with PetJ’s assignment as the immediate electron donor to P800+ based on the identical optical kinetics of a cytochrome c species with a midpoint potential +190 mV and P800+. Furthermore, when an inhibitor of the Cyt b subunit of the Cyt bc complex, stigmatellin, was added to whole cells and membranes of *H. gestii*, re-reduction of PetJ was strongly inhibited, implying that it functions as the intervening electron shuttle to P800+. The almost identical optical properties of the cytochromes in heliobacteria, in combination with the fact that PetJ does not copurify with the RC, makes quantification of the stoichiometry of PetJ to HbRC in heliobacterial cells difficult. *In vivo* optical experiments on whole cells of *H. chlorum* and *H. mobilis* suggest the ratio to be anywhere between 1-6 cytochromes per RC. Whether or not this value reflects the PetJ to HbRC ratio exclusively, with no contribution from other c-type cytochromes, is still uncertain.
Interestingly, the rate of ET between the membrane anchored PetJ cytochrome and P₈₀₀⁺ has been shown to slow by at least an order of magnitude, from 100 to 700 μsec in vivo to 3-6 ms, after disruption of the cellular membrane⁸⁹, ⁹³-⁹⁵. In combination with the significant temperature (Eₐ = 88.2-91 kJ/mol) dependence on the ET rate, as well as the observation that PetJ does not copurify with the RC, suggest that the physical interaction between PetJ and the RC is collisional²⁵, ⁹², ⁹₅.

1.5.2 Green sulfur bacteria: integral membrane cytochrome PscC

In contrast to the lipid-anchored PetJ, most GSbRCs contain two PscC cytochromes (Cyt c₅₅₁, where 551 is the wavelength of maximal bleaching) that copurify with the RC and function as the immediate donor to P₈₄₀⁺⁶⁶, ⁹⁷-⁹⁹. This membrane bound cytochrome contains an N-terminal domain consisting of three transmembrane α helices and a C-terminal domain that binds the monoheme ET cofactor via the highly conserved c-type cytochrome heme binding motif CxxCH¹⁰⁰. Interestingly, PscC does not copurify with the GSbRC from Prosthecochloris aestuarii¹⁰¹. The reason for this discrepancy is unknown but it may be related to a 52 amino acid insertion between the N- and C-terminal domains found in the PscC sequences from Prosthecochloris species¹⁷.
The kinetics of forward ET from the PscC subunits of the GSbRC have been measured in purified RCs to be either biphasic, with $t_{1/2}$ values of 7-10 μsec and 50-60 μsec in *C. tepidum* and *C. vibrioforme*, or monophasic with a $t_{1/2}$ of either 110 μsec or 28 μsec in *C. tepidum*\textsuperscript{64,98,100,102}. Similar to PetJ in heliobacteria, the redox potential of PscC was measured to be +170-180 mV (v. SHE), providing a small but favorable 70-80 meV driving force for the reduction of the primary donor P\textsubscript{940*} ($E_m = +240$-$250$ mV)\textsuperscript{39,46,103}.

Including PscC, there are 11 total c-type cytochromes annotated in the *C. tepidum* genome\textsuperscript{7}. These include a soluble Cyt c\textsubscript{553/554} that has been shown to reduce PscC and may serve as an electron shuttle from some of the other monoheme c-type cytochromes such as SoxX and SoxA involved in the oxidation of thiosulfate\textsuperscript{7,104,105}. Additionally, a gene encoding a putative Cyt c\textsubscript{556} is present that is similar to PetJ in heliobacteria. Cyt c\textsubscript{556} is predicted to be posttranslationally modified at Cys 18 by the covalent attachment of a diacylglycerol moiety that would anchor the cytochrome to the membrane via fatty acids\textsuperscript{17}. This putative membrane-anchored Cyt c\textsubscript{556} has been shown to directly accept electrons from a Cyt b/Rieske-type complex and reduce PscC, thereby connecting the reduced quinone pool with photosynthetic ET. In this manner Cyt c\textsubscript{556} may functionally substitute for the missing Cyt c\textsubscript{1}. 


28
protein (encoded by petC) present in the Cyt bc complex of other bacteria.5

1.5.3 Chloracidobacterium thermophilum cytochromes

The only experimental evidence suggesting the participation of cytochromes in the RC in C. thermophilum comes from the observations of Tsukatani et al. who noted 1) an optical photobleaching at 553 nm in whole cells and 2) the recovery of steady-state photobleached P840+ in membrane fragments was accelerated by the addition of soluble protein fractions from cell lysates. These results suggest that a soluble component can act directly or indirectly as an electron donor to P840+. Isolated RC complexes showed no presence of a tightly associated cytochrome, an observation consistent with the lack of a pscC gene in the pscA-pscB-fmoA operon in the genome of C. thermophilum. Although five putative c-type cytochromes are encoded in the genome, no biochemical data exists to support their role in photosynthetic ET.

Similar to GSB, both Cyt bc complex operons (pet) are lacking a petC gene that encodes the Cyt c1 subunit. However, just as Cyt c556 may substitute for the missing Cyt c1 in GSB, both petAB operons in C. thermophilum have been suggested to contain plausible alternatives for the missing Cyt c1.
1.6 Dissertation Overview

All homodimeric Type I RCs convert sunlight into stored chemical potential in the form of reduced FeS clusters. The thermodynamic and kinetic properties of the reactions that generate these low-potential electron acceptors, however, are not well understood. Chapter 2 will describe the first complete thermodynamic characterization of a homodimeric ET chain by direct measurement of the midpoint potentials of $F_X$, $F_A$, and $F_B$ in the HbRC. Theoretical treatment of the ET chain with a quasi-equilibrium model of charge recombination estimates the midpoint potential of the primary electron acceptor, $A_0$. In Chapter 3, a kinetic characterization of the ET chain in chlorosome-depleted membranes of the aerobic bacterium *C. thermophilum* provides evidence of an ET pathway similar to the HbRC and GSbRC. In contrast, PscB is bound tighter to the CabRC core than the analogous PscB ferredoxin in GSbRCs. Under reducing conditions, charge recombination via the $^3P$ is observed in all homodimeric RCs. Due to the zero-field splitting parameters for BChl $g^\prime$ ($D \approx 3E$) $^3P_{800}$ can only be observed optically or in oriented samples. In Chapter 4, the effect of temperature, light, and oxygen exposure on the oxidation of BChl $g$ is studied with respect to energy and ET. The loss of HbRC activity as a function of pigment oxidation is nonlinear and cannot be explained by sub-saturating light effects associated
with a reduction of antenna size alone. The hypothesis is presented that charge-separation and ET proceed in oxidized RCs containing a functional heterodimeric special pair consisting of BChl $g'$/Chl $a'_{ox}$. 
1.7 References


photovoltage studies on chlorosome containing bacteria, Photosynth.
Res. 58, 57-70.
(1998) Transient electron paramagnetic resonance spectroscopy on
green sulfur bacteria and heliobacteria at two microwave
and primary photochemical reactions in the photosynthetic
cellulose Helio bacterium chlorum, Proc. Natl. Acad. Sci. U.S.A. 82,
6865-6868.
Excited states and primary charge separation in the pigment system
of the green photosynthetic bacterium Prosthecochloris aestuarii as
studied by picosecond absorbance difference spectroscopy, Biochim.
Biophys. Acta 807, 24-34.
[14] Redding, K. E., Sarrou, I., Rappaport, F., Santabarbara, S., Lin, S., and
Reifschneider, K. T. (2014) Modulation of the fluorescence yield in
heliobacterial cells by induction of charge recombination in the
photosynthetic reaction center, Photosynth. Res. 120, 221-235.
separation and formation of bacteriochlorophyll triplets in
[16] Bochove, A. C. v., Swarthoff, T., Kingma, H., Hof, R.M., Grondelle, R.
charge separation in green bacteria by means of flash spectroscopy,
cytochromes in the photosynthetic electron transfer pathways in
green sulfur bacteria and heliobacteria, Photosynth. Res. 104, 189-
199.
serves as the terminal bound electron acceptor in heliobacterial
[19] Seo, D., Tomioka, A., Kusumoto, N., Kamo, M., Enami, I., and Sakurai,
reaction center complex from the green sulfur bacterium Chlorobium


photosynthetic reaction center of *Heliobacterium modesticaldum*, Biochemistry 44, 9950-9960.


1.8 Figures

Figure 1.1: Cartoon representation of the RC complexes and bound electron transfer cofactors from (A) heliobacteria, (B) green sulfur bacteria, and (C) chloracidobacteria. Note that no cytochrome is indicated for chloracidobacteria since the identity of the secondary electron donor
responsible for reduction of $P_{840}^*$ has not been confirmed. See text for details.

Abbreviations: FMO – Fenna-Matthews-Olson complex, CBP – carotenoid binding protein, Cyt $c_{553}$ – c-type cytochrome encoded by petJ.
1.9 Tables

### RC Complex Composition

<table>
<thead>
<tr>
<th>Function</th>
<th>heliobacteria</th>
<th>green sulfur bacteria</th>
<th>chloracidobacteria</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC Core</td>
<td>PshA</td>
<td>PscA</td>
<td>PscA</td>
<td>binds ET chain and antenna</td>
</tr>
<tr>
<td>Terminal Acceptors</td>
<td>PshBII/PshBII</td>
<td>PscB</td>
<td>PscB</td>
<td>Fs and Fs FeS protein</td>
</tr>
<tr>
<td>Secondary Donor</td>
<td>Cyt c551 (Psh)</td>
<td>Cyt c551 (Psc)</td>
<td>?</td>
<td>PshE membrane anchored via fatty acids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PscC-TM α helices</td>
</tr>
<tr>
<td>Light Harvesting</td>
<td>–</td>
<td>Chlorosomes</td>
<td>Chlorosomes</td>
<td></td>
</tr>
<tr>
<td>Energy Transfer</td>
<td>–</td>
<td>FM0</td>
<td>FM0</td>
<td>Fenna-Matthews-Olson</td>
</tr>
<tr>
<td>Energy Transfer/Fd Reduction</td>
<td>–</td>
<td>PscD</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Photoprotection/Antenna</td>
<td>–</td>
<td>–</td>
<td>Cbp</td>
<td>carotenoid binding protein</td>
</tr>
</tbody>
</table>

### Electron Transfer Chain

<table>
<thead>
<tr>
<th>Function</th>
<th>heliobacteria</th>
<th>green sulfur bacteria</th>
<th>chloracidobacteria</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Donor</td>
<td>BChl g'/g'</td>
<td>Mg-BChl a'/a'</td>
<td>Zn-BChl d'/d'</td>
<td>Prime denotes the C-13 epimer.</td>
</tr>
<tr>
<td>Em vs. SHE</td>
<td>+225 mV</td>
<td>+240-250 mV</td>
<td></td>
<td>Pos of GSB actually displays maximal photobleaching at 830 nm</td>
</tr>
<tr>
<td>A0</td>
<td>8'-OH Chl a*</td>
<td>Chl a27,28**</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Em vs. SHE</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>menaquinone7</td>
</tr>
<tr>
<td>A2/Fx</td>
<td>[4Fe4S]2+/1+</td>
<td>[4Fe4S]2+/1+</td>
<td>[4Fe4S]2+/1+</td>
<td></td>
</tr>
<tr>
<td>Spin State</td>
<td>S = 3/2</td>
<td>S = 3/2</td>
<td>S = 3/2</td>
<td></td>
</tr>
<tr>
<td>Em vs. SHE</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>Fx/Fb</td>
<td>2[4Fe4S]1+/1+</td>
<td>2[4Fe4S]1+/1+</td>
<td>2[4Fe4S]1+/1+</td>
<td></td>
</tr>
<tr>
<td>Em vs. SHE</td>
<td>?</td>
<td>-441 mV/-501 mV</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1: Summary of homodimeric RC complex subunits (top) and ET chain cofactor identities and properties (bottom). For References, see: a, Prince et al. (1985)\textsuperscript{46}; b, Fowler et al. (1971)\textsuperscript{39}; c, Prince et al. (1976)\textsuperscript{46}; d, Heinnickel et al. (2006)\textsuperscript{63}; e, Jagannathan and Golbeck (2008)\textsuperscript{64}; f, Scott et al.
Chapter 2

Thermodynamics of the Electron Acceptors in *Heliobacterium modesticaldum*: An Exemplar of an Early Type I Photosynthetic Reaction Center

Published as:
2.1 Abstract

The homodimeric Type I reaction center in heliobacteria is arguably the simplest known pigment-protein complex capable of carrying out (bacterio)chlorophyll-based conversion of light into chemical energy. Despite its structural simplicity, the thermodynamics of the electron transfer cofactors on the acceptor side have not been fully investigated. In this work, we measured the midpoint potential of the terminal [4Fe-4S]^{2+/1+} cluster (F_X) in reaction centers from *Helio bacterium modesticaldum*. The F_X cluster was titrated chemically and monitored by (i) the decrease of stable P_{800} photobleaching by optical spectroscopy, (ii) the loss of the light-induced g \approx 2 radical from P_{800}^+ following a single-turnover flash, (iii) the increase in the low-field resonance at 140 mT attributed to the S = 3/2 ground spin state of F_X^-, and (iv) the loss of the spin-correlated P_{800}^+F_X^- radical pair following a single-turnover flash. These four techniques led to similar estimations of the midpoint potential for F_X of -502 \pm 3 mV (n = 0.99), -496 \pm 2 mV (n = 0.99), -517 \pm 10 mV (n = 0.65), and -501 \pm 4 mV (n = 0.84), respectively, with a consensus value of -504 \pm 10 mV (converging to n = 1). Under conditions in which F_X is reduced, the long-lived (~15 ms) P_{800}^+F_X^- state is replaced by a rapidly recombining (~15 ns) P_{800}^+A_0^- state, as shown by ultra-fast optical experiments. There was no evidence for the presence of a P_{800}^+A_1^- spin-
correlated radical pair by EPR under these conditions. The midpoint potentials of the two [4Fe-4S]$^{2+/1+}$ clusters in the low molecular mass ferredoxins were found to be $-480 \pm 11$ mV/$-524 \pm 13$ mV for PshBI, $-453 \pm 6$ mV/$-527 \pm 6$ mV for PshBII and $-452 \pm 5$ mV/$-533 \pm 8$ mV for HM1_2505 by EPR spectroscopy. $F_X$ is therefore suitably poised to reduce one [4Fe-4S]$^{2+/1+}$ cluster in these mobile electron carriers. Using the measured midpoint potential of $F_X$ and a quasi-equilibrium model of charge recombination, the midpoint potential of $A_0$ was estimated to be $-854$ mV at room temperature. The midpoint potentials of $A_0$ and $F_X$ are therefore 100 to 200 mV more oxidizing than their respective counterparts in Photosystem I of cyanobacteria and plants. This places the redox potential of the $F_X$ cluster in heliobacteria approximately equipotential to the highest potential iron-sulfur cluster ($F_A$) in Photosystem I, consistent with its assignment as the terminal electron acceptor.

### 2.2 Introduction

Bacteriochlorophyll (BChl) and chlorophyll (Chl)-based photosynthetic reaction centers (RCs) are membrane-bound, pigment-protein complexes that catalyze the conversion of light into chemical energy. Photosynthetic RCs are found in the bacterial phyla: *Cyanobacteria*, *Proteobacteria* (purple sulfur and nonsulfur bacteria), *Firmicutes*
(heliobacteria), Chlorobi (green sulfur bacteria), Chloroflexi (filamentous anoxygenic phototrophs), Acidobacteria (chloracidobacteria)\textsuperscript{1,2}. Based on the identity of the terminal electron acceptor, photosynthetic RCs can be subdivided into two broad classes. In Type I RCs, the terminal acceptor is a [4Fe-4S] cluster and in Type II RCs, the terminal acceptor is a mobile quinone. Cyanobacteria, algae and plants contain a heterodimeric Type I RC termed Photosystem I (PSI), whereas heliobacteria, green sulfur bacteria and acidobacteria contain a homodimeric Type I RC. Even though all known photosynthetic RCs contain the same basic architecture of a bifurcated electron transfer chain, phylogenetic analyses indicate that PsaA and PsaB, the two core polypeptides of PSI, are much more closely related to each other than to the core polypeptide of homodimeric Type I RCs\textsuperscript{3}. Of the anoxygenic Type I RCs, the heliobacterial RC (HbRC) may more closely resemble the common ancestor of all RCs due to its simple polypeptide and cofactor composition\textsuperscript{3-7}.

Photosynthetic RCs operate at quantum efficiencies that approach unity by efficiently transferring the excited state among the antenna Chls to the trapping center and by stabilizing the oxidized donor/reduced acceptor pair through a series of rapid and energetically favorable electron transfer steps. The HbRC binds \(~18\) BChl\textsubscript{g} molecules that serve as antenna, two BChl
g′molecules (the C-13² epimer of BChl g) that constitute the special pair P₈₀₀, two 8¹-OH Chl αF molecules that serve as the primary electron acceptor(s) A₀, an interpolyptide [4Fe-4S]²⁺/¹⁺ cluster Fₓ, one C30 carotenoid diaponeurosporene, and up to two molecules of menaquinone (MQ-8/MQ-9)⁶.⁸.⁹. Assuming the HbRC follows the same motif as PSI, two of the ~18 BChl g molecules will function as accessory pigments between P₈₀₀ and A₀, consistent with a recent analysis of ultra-fast decay-associated difference spectra and the P₈₀₀⁺ – P₈₀₀ difference spectrum¹⁰. Following photon absorption, the P₈₀₀⁺A₀⁻ charge-separated state is generated within ~25 ps¹⁰. The lifetime of A₀⁻ is 600 to 800 ps¹⁰.¹¹, after which the electron is passed to a secondary acceptor. In the presence of a strong reductant, the lifetime of A₀⁻ is extended to ~17 ns and it decays concomitantly with the loss of P₈₀₀⁺⁹.¹² (i.e. charge recombination of P₈₀₀⁺A₀⁻), thereby destroying the charge-separated state. In the absence of a strong reductant, the electron on A₀ is transferred to a secondary acceptor. If the electron transfer chain is similar to PSI, the two molecules of MQ present in the HbRC would function in a manner analogous to the phylloquinones of PSI¹³-¹⁷. Evidence supporting the role of MQ in forward transfer chain includes the observation of a radical attributed to a reduced semiquinone detected by transient and CW EPR spectroscopy¹⁸.¹⁹. Indirect evidence of a secondary quinone acceptor
between $A_0$ and $F_X$ comes from the $E_m = -414$ mV ($n = 2$) value measured by optically monitoring the photobleaching of $P_{800}^+$ in isolated HbRCs$^{20}$ and attributed to a doubly reduced MQ. Nevertheless, there is equally compelling evidence against the role of MQ in forward electron transfer, in particular, the finding that the MQ is not tightly bound to the HbRC homodimeric core and its removal by solvent extraction has no effect on the rate of charge recombination between secondary acceptors and $P_{800}^{+12}$. This assessment is supported by time-resolved optical, certain transient EPR, and photovoltage measurements that showed no evidence for the presence of a cofactor between $A_0$ and $F_X^{15-17}$. Therefore, in spite of the structural similarity between phylloquinone and MQ, the role of the latter in the HbRC remains uncertain.

The $F_X$ cluster had been first proposed to exist in a $S = \frac{1}{2}$ ground spin state with a rhombic set of resonances at $g(z)=2.040$, $g(y)=1.911$, and $g(x)=1.896^{21}$. However these signals were only observed when the PshBI protein was bound and the $F_A$ and $F_B$ clusters were chemically reduced; they were not observed in HbRC cores that were depleted of PshBI. A prominent set of low-field resonances around 140 mT were later discovered in HbRC cores and interpreted as the ground and excited state Kramer’s doublets of a $S = 3/2$ spin state $F_X$ cluster$^8$. Mössbauer spectra supported this assignment
by showing that the Fe in the as-isolated HbRC is present in the form of a
$[4\text{Fe-4S}]^{2+}$ cluster which is converted to a $[4\text{Fe-4S}]^{1+}$ cluster with a $S = 3/2$
ground spin state following photoreduction$^8$.

Because of the uncertainty in the role and composition of the electron
acceptors, a quantitative description of their thermodynamic properties in
homodimeric Type I RCs is lacking. In this study we measured the midpoint
potential of the terminal acceptor in isolated HbRCs by monitoring four
observables as a function of solution potential: (i) the decrease of stable $P_{800}$
photobleaching by optical spectroscopy, (ii) the loss of the light-induced $g \approx 2$
radical from $P_{800}^+$ following a single-turnover flash, (iii) the increase in the
low-field resonance at 140 mT of the $S = 3/2$ ground spin state of $F_{X^-}$, and (iv)
the loss of the spin-correlated $P_{800}^+$ $F_{X^-}$ radical pair following a single-
turnover flash. We also measured the midpoint potentials of three low
molecular mass dicluster ferredoxins in $H. modesticaldum$, PshBI, PshBII and
HM1_2505, by EPR spectroscopy. We use the measured midpoint potential of
$F_{X}$ and a quasi-equilibrium model of charge recombination at room
temperature to estimate the midpoint potential of $A_0$. The experimentally
determined midpoint potentials of $A_0$ and $F_{X}$ as well as the midpoint
potentials of PshBI, PshBII and HM1_2505, reveal a marked difference in the
energetics of the acceptor chain of this anoxygenic RC compared with PSI, its oxygenc counter part in plants and cyanobacteria.

2.3 Materials and Methods

2.3.1 Growth of Heliobacterium modesticaldum and preparation of membranes

Cultures of Heliobacterium modesticaldum were grown anoxically in PYE media with the addition of resazurin (0.001%) as an oxygen indicator, as previously described \(^8,^{22,23}\). Cells were grown to late exponential phase and harvested anoxically by centrifugation at 10,000 x g. The cells were resuspended in 50 mM MOPS pH 7.0 and lysed via sonication inside an anoxic chamber (Coy Labs, Grass Lake, MI). All manipulations were carried out under dim light and under strict anoxic conditions. Cell membranes were collected by centrifugation at 200,000 x g and resuspended in 50 mM MOPS, pH 7.0.

To open liposomes and remove extrinsic proteins, membranes were resuspended to an OD\(_{788\text{ nm}}\) of 1-2 in 100 mM sodium carbonate (pH 11.5) buffer, incubated for 1 hour in the dark, and harvested by centrifugation at 200,000 x g for 1 hour\(^{24}\). Membrane pellets were resuspended in the same buffer and incubated for an additional hour in the dark before being
harvested and resuspended in in a buffer containing 50 mM MES (pH 6.0) and 20 mM MgSO₄ at a BChl \textit{g} concentration of 3 mM.

2.3.2 Isolation of heliobacterial reaction centers

Membranes (without carbonate pre-treatment) were solubilized with 1% \textit{n}-dodecyl-\textit{\beta}-\textit{D}-maltopyranoside (DDM, Anatrace, Maumee, OH) for one hour at room temperature after dilution to a final concentration of 0.5 mM BChl \textit{g}. The latter was determined by the absorbance at 788 nm using an extinction coefficient for BChl \textit{g} of 110 mM⁻¹ cm⁻¹. Insoluble material was removed from the solution by centrifugation at 200,000 x g. The soluble supernatant was passed over a diethylaminoethyl (DEAE-Sepharose Fast Flow) anion exchange column (Sigma Aldrich, St. Louis, MO) equilibrated with 50 mM MOPS pH 7.0 and 0.02% DDM. The brown flow-through fraction enriched in HbRCs was concentrated using N₂ gas (60 psi) in a pressurized Amicon stir-cell (EMD Millipore, Darmstadt, Germany) over a 30-kDa polyethersulfone membrane (Pall, Port Washington, NY).

2.3.3 Redox potentiometry of the HbRC by optical spectroscopy

HbRC samples were prepared in buffers ranging from pH 6.0-10.0 in the presence of 10 mM ascorbate and 0.02% DDM. MES was used for pH 6.0-6.5, MOPS was used for pH 6.6-7.9, Tricine was used for pH 8.0-8.9, and Glycine was used for pH 9.0-10.0. Laser-flash induced photobleaching was
measured at 800 nm on the JTS-10 (Bio-Logic) following a single pulse of actinic light of 25 mJ at 532 nm with a pulse width of 6 ns provided by a frequency doubled Minilite-II Nd:YAG laser (Continuum, San Jose, CA). The samples were then treated with 10 mM dithionite in the dark for up to 3 hours. Photobleaching measurements were taken every 15 minutes until the amplitude of the bleaching at 500 µs after the flash remained constant between measurements. The fraction reduced was calculated by dividing the amplitude of the bleaching for the dithionite treated sample by the amplitude measured prior to addition of dithionite. The ambient reduction potential of the various solutions were measured in the absence of the HbRC with 50 µM of benzyl viologen (−359 mV/SHE)\textsuperscript{26}, methyl viologen (−446 mV/SHE)\textsuperscript{26}, 1,1′-trimethylene-2,2′-dipyridinium dibromide (−552 mV/SHE)\textsuperscript{27} and 4,4′-dimethyl-1,1′-trimethylene-2,2′-dipyridinium dibromide (−691 mV/SHE)\textsuperscript{27} using a saturated calomel electrode and platinum counter-electrode.

2.3.4 Ultra-fast transient absorption spectroscopy

Transient absorption measurements were performed at room temperature using a broad-band pump-probe setup described previously\textsuperscript{28}. Laser pulses (800 nm) of 100 fs duration were generated from a regenerative amplifier system (Tsunami and Spitfire, Spectra-Physics) at a repetition rate of 1 kHz. Part of the beam was used to pump an optical parametric amplifier
(IR OPA, Spectra-Physics) to generate 575-nm pulses for sample excitation. The broadband probe pulse was generated by focusing the 800 nm beam into a 3 mm sapphire plate, and was sent to an optical compressor composed of a pair of prisms, before being focused on the sample. The white-light probe pulses were then dispersed by a spectrograph and detected with a CCD camera (DU420, Andor Technology). Collected data had a spectral resolution of 1.17 nm. The polarization of the pump pulses was set to the magic angle (54.7°) with respect to that of the probe pulses. The sample of HbRC that contained 10 mM ascorbate and 5 µM PMS was loaded into a spinning wheel with an optical path-length of 1.2 mm. The sample that contained 10 mM dithionite was loaded into a sealed quartz cuvette with an optical path-length of 2 mm. The OD_{788} of the samples were adjusted to 0.9 in the corresponding sample cell used.

Decay-associated difference spectra (DADS) were calculated from global fitting after accounting for deconvolution of the recorded signals with an instrument response function using locally written software, ASUFIT (www.publicasu.edu/laserweb/asufit/asufit.html). The instrument response function was fit to a Gaussian curve (full-width at half maximum of 100–150 fs). The absorption changes as a function of time (t) and probe wavelength
(λ) were fit with a multi-exponential kinetic model from 620 – 850 nm simultaneously using the equation

$$\Delta A(\lambda, t) = \sum_i a_i(\lambda) e^{-\frac{t}{\tau_i}}$$

where \(\tau_i\) is the decay lifetime of the \(i\)th decay component and \(a_i\) is the wavelength-dependent amplitude of this component.

2.3.5 EPR spectroscopy on carbonate-washed membranes

Carbonate-washed membranes were treated with 30 mM dithionite at pH 6.0 or 10.0 (see results) for 30 minutes in the dark. Illumination of EPR samples was performed using a 1000 W Oriel quartz-tungsten halogen lamp (Model 6405) passed through a copper sulfate solution to give a light intensity of 750 \(\mu\text{E/m}^2/\text{s}\) at room temperature for 30 seconds. Following illumination, samples were given a 2 second period of darkness prior to freezing in a dry ice ethanol bath. Samples were stored in the dark in liquid nitrogen prior to measurement.

EPR measurements were performed with a Bruker E580 X-band spectrometer (Bruker BioSpin Corp., Billerica, MA) with an Oxford Model 900 EPL liquid helium cryostat (Oxford Instruments, Abingdon, UK). All spectra were measured with a modulation frequency of 100 kHz and modulation
amplitude of 1 mT. The microwave power used was 0.25 mW with a frequency of approximately 9.43 GHz unless otherwise noted.

Fitting of EPR spectra was performed using EasySpin (version 3.0.0)\textsuperscript{29}. The fitting parameters used were the three $g$ values ($g_x$, $g_y$, and $g_z$), the corresponding line widths ($\Delta B_x$, $\Delta B_y$, $\Delta B_z$), and the zero-field splitting parameters ($D$ and $E$). The fitting method used here was similar to those previously described by Flores et al\textsuperscript{30}.

2.3.6 Redox potentiometry of the HbRC by EPR spectroscopy

Concentrated HbRCs stored in liquid nitrogen were thawed in the dark under strictly anoxic conditions and diluted with a solution of 1 M glycine pH 10.0 containing 100 µM of benzyl viologen (−359 mV/SHE), methyl viologen (−446 mV/SHE), 1,1’-trimethylene-2,2’-dipyridinium dibromide (−552 mV/SHE) and 4,4’-dimethyl-1,1’-trimethylene-2,2’-dipyridinium dibromide (−691 mV/SHE) as electron mediators ([BChl $g$]$_{\text{final}} = 3$ mM)\textsuperscript{26, 27, 31}. A pH meter was used to ensure a final pH of 10.0. The solutions were continuously stirred in a custom built glass titration cell and the solution potential was measured with a high impedance digital voltmeter across a gold working electrode (Sigma) and a Ag/AgCl reference electrode (MI-402) (Microelectrodes, Bedford, NH). The reference electrode was calibrated using a saturated solution of quinhydrone, which
disproportionates to a 1:1 mixture of benzoquinone and benzohydroquinone at pH 7.0, and the potentials corrected for temperature in the conversion from Ag/AgCl to standard hydrogen electrode (SHE). After the addition of a small aliquot of concentrated sodium dithionite solution (300 mM in 1M glycine pH 10.0), the solution potential was allowed to stabilize (ca. 5 min), and the sample was transferred to a 4 mm O.D. X-band EPR tube (Wilmad, Warminster, PA) and frozen inside the anoxic chamber in liquid nitrogen. Samples were stored in liquid nitrogen until measurement.

The amplitude of the resonance at 140 mT from $F_X$ was measured using a Bruker E500 EPR spectrometer (ELEXSYS, Bruker BioSpin Corp., Billerica, MA) at X-Band (9.39 GHz). A cylindrical TE 011-mode resonator (Bruker BioSpin Corp., Billerica, MA) was used and the temperature was maintained at 4.2 K with an ESR 900 liquid helium cryostat and an ITC-4 controller (Oxford Instruments, Abingdon, UK). The sample was placed in the EPR cavity in darkness and the signal height at 140 mT was plotted against solution potential to obtain the midpoint potential of $F_X$.

The amplitude of the light-induced $g \approx 2$ signal from $P_{800}^+$ was measured using a Bruker E300 EPR spectrometer at X-band using field modulation. A Flexline dielectric resonator (ER 4118 X-MD-5W1, Bruker BioSpin Corp., Billerica, MA) was used and the temperature was maintained
at 90 K with an ER 4118CF liquid helium cryostat (Bruker BioSpin Corp., Billerica, MA) and an ITC-4 controller. Time-sweep measurements of the lifetime of $P_{800}^\cdot$ at 90 K were carried out using a Bruker E300 EPR spectrometer at X-band using field modulation. The field position of the low-field peak of the steady-state $P_{800}^\cdot$ radical was determined by first measuring the light-minus-dark difference spectrum using continuous illumination provided by a Millennia V CW diode pumped Nd:YVO$_4$ laser (Spectra Physics, Santa Clara, CA) at 532 nm and 100 mW (CW, EPR mode). The spectrometer was then switched to time-sweep mode and the magnetic field fixed to the field position of the low-field peak as determined from the difference spectrum. The sample was interrogated by single-turnover actinic flashes at a repetition rate of 0.5 Hz provided by a frequency doubled Vibrant 355Il Nd:YAG laser at 532 nm with a single pulse energy of 20 mJ and a pulse width of 7 ns (OPOTEK Inc, Carlsbad, CA). Data represent the average of 512 spectra and lifetimes were determined by using a nonlinear least squares multi-exponential fitting algorithm in Igor Pro (Igor Pro, Lake Oswego, OR). The maximum amplitude of the transient flash induced $P_{800}^\cdot$ radical was plotted as a function of solution potential to determine the midpoint potential of F$_X$. 
The amplitude of the light-induced spin-polarized radical $P_{800}^+ F_X^-$ was measured using a modified Bruker E300 EPR spectrometer at X-band in direct detection mode. A Flexline dielectric resonator (ER 4118 X-MD-5W1, Bruker BioSpin Corp., Billerica, MA) was used and the temperature was maintained at 90 K with an ER 4118CF liquid helium cryostat (Bruker BioSpin Corp., Billerica, MA) and an ITC-4 controller. The sample was placed in the EPR cavity in darkness. The transient EPR signal was collected with a home-built broadband amplifier (bandwidth >500 MHz), and digitized using a 500 MHz bandwidth, 8-bit, 2GS/s, Model CS85G PCI card (DynamicSignals LLC, Lockport, IL). The software for controlling the spectrometer and for data acquisition was written in-house in LabView. Three dimensional time/field/amplitude data sets were collected following single-turnover flashes at 20 mJ per pulse from a Vibrant 355II laser (Opotek, Inc., Carlsbad, CA) running at 10 Hz and a wavelength of 532 nm with a pulse width of 7 ns. Each time/field data set consists of 128 transient EPR signals collected and averaged at each fixed magnetic field at a resolution of 25 µT. Boxcar spectra were extracted from the field/time data sets by integrating the EPR signal between 1-3 µsec following the laser flash. The signal height at 346.52 mT was plotted from the amplitude v. magnetic field slice against solution potential to estimate the midpoint potential of $F_X$. 
The titration data was fit to the Nernst equation (a) using a nonlinear least squares algorithm in Igor Pro (Igor Pro, Lake Oswego, OR).

\[
(a) \quad [\text{red}] = \frac{\text{max intensity}}{\left(\frac{E - E_m}{0.05}\right)^n + 1}
\]

2.3.7 Cloning, growth and purification of PshBI, PshBII and HM1_2505 in Escherichia coli

The genes encoding the PshBI, PshBII and HM1_2505 proteins were codon optimized for E. coli and synthesized (GenScript, Piscataway, NJ) for cloning into pET28b (EMD Millipore, Darmstadt, Germany) between the NdeI and BamHI restriction sites, resulting in a N-terminal fusion with a hexahistidine tag. The pET28b plasmids containing the ferredoxin genes were transformed into a BL21(DE3) strain with a deletion of the iron-sulfur cluster biosynthesis repressor (iscR). Strains were grown in LB media supplemented with 100 mM MOPS buffer and the pH was adjusted to 7.4 before inoculation. Cells were grown in the presence of 2 mM L-Cys (Sigma Aldrich, St. Louis, MO), 2 mM ferric ammonium citrate (ICN Biomedicals, Irvine, CA), 25 mM glucose, 25 mM sodium fumarate and 50 µg/mL kanamycin (Sigma Aldrich, St. Louis, MO) in a 3 L spinner flask (Chemglass, Vineland, NJ) as described by Kuchenreuther et al\textsuperscript{32}. Cultures were induced with 200 µM IPTG (Goldbiotech, St. Louis, MO) and were grown under
continuous stirring and bubbling with argon gas for 20 hours at room temperature. All manipulations were carried out under strict anoxic conditions. Cells were harvested by centrifugation at 10,000 x g and resuspended in 50 mM MOPS pH 7.0 with 100 mM NaCl. Cells were lysed by sonication inside an anoxic chamber (Coy Labs, Grass lake, MI) and insoluble material and unbroken cells were removed by centrifugation at 27,000 x g. The supernatant was cleared of the membrane component by ultracentrifugation at 235,000 x g. The cleared lysate was loaded onto a pre-equilibrated (50 mM Tris HCl pH 8.0, 100 mM NaCl) Ni-NTA column (Macherey-Nagel, Bethlehem, PA). The column was washed with 20 column volumes of 50 mM Tris HCl pH 8.0 with 100 mM NaCl and 40 mM imidazole. The ferredoxins were eluted with 50 mM Tris HCl pH 8.0, 100 mM NaCl and 300 mM imidazole. Samples were wash concentrated using a 3 kDa regenerated nitrocellulose cutoff membrane (Pall, Port Washington, NY) under nitrogen gas (60 psi) in a pressurized Amicon stir cell (EMD Millipore, Darmstadt, Germany) followed by a buffer exchange with a PD-10 (GE Healthcare, Little Chaufont, UK) size exclusion column equilibrated with 50 mM Tris HCl pH 8.0 to remove the NaCl and imidazole.

2.3.8 Redox potentiometry of PshBI, PshBII and HM1_2505 by EPR spectroscopy
Redox titrations of PshBI, PshBII and HM1_2505 were carried out as described for all titrations with the HbRC above using dithionite at pH 10.0 and a cocktail of electron mediators. Titrations were carried out under strict anoxic conditions and at room temperature. EPR samples were frozen inside the anoxic chamber and stored under liquid nitrogen until EPR measurements were made. For PshBI and PshBII, EPR spectra were collected using a Bruker E300 EPR spectrometer at X-band using field modulation. A Flexline dielectric resonator (ER 4118 X-MD-5W1, Bruker BioSpin Corp., Billerica, MA) was used and the temperature was maintained at 15 K with an ER 4118CF liquid helium cryostat (Bruker BioSpin Corp., Billerica, MA) and an ITC-4 controller. HM1_2505 was measured using a Bruker E500 EPR spectrometer (ELEXSYS, Bruker BioSpin Corp., Billerica, MA) at X-Band. A cylindrical TE 011-mode resonator (Bruker BioSpin Corp., Billerica, MA) was used and the temperature was maintained at 15 K with an ESR 900 liquid helium cryostat and an ITC-4 controller (Oxford Instruments, Abingdon, UK). Double integrations of the resulting EPR spectra were plotted against the solution potential measured with a high impedance digital voltmeter across a gold working electrode (Sigma) and a Ag/AgCl reference electrode (MI-402) (Microelectrodes, Bedford, NH). As for all titrations of the HbRC, the measured solution potential was calibrated using a saturated solution of
quinhydrone at pH 7.0. The resulting titration curves were fit as previously described by Scott et al. using a modified form of the Nernst equation (b) that assumes two independent redox centers with different midpoint potentials\textsuperscript{33}.

\[
(b) \quad [\text{red}] = \frac{\text{max intensity}}{10^{\frac{E - E_{m1}}{\Delta E}}} + 1 + \frac{\text{max intensity}}{10^{\frac{E - E_{m2}}{\Delta E}}} + 1
\]

2.4 Results

2.4.1 Time-resolved optical studies of P\textsubscript{800} photobleaching in HbRCs reduced by dithionite

Excitation of isolated HbRCs with a laser flash results in a bleaching centered at \(~800\) nm due to formation of the P\textsubscript{800}\textsuperscript{+}F\textsubscript{X}\textsuperscript{–} charge-separated state. In the absence of a donor or acceptor, P\textsubscript{800}\textsuperscript{+}F\textsubscript{X}\textsuperscript{–} undergoes charge recombination with a lifetime of \(~15\) ms, resulting in decay of the bleaching at 800 nm\textsuperscript{8} (Figure 2.1A). Treatment of HbRCs with sodium dithionite at pH 10.0 in the dark results in the loss of \(>90\%\) of the P\textsubscript{800} bleaching signal on a single flash. This effect can be reversed by a brief treatment with air: merely uncapping the cuvette and allowing air to mix gently with the solution by inversion results in restoration of the light-induced P\textsubscript{800} signal (Figure 2.1A). This effect was reversible: after recapping the cuvette and allowing it remain in the dark for several minutes, the light-induced signal disappeared. The cycle of disappearance and reappearance of the P\textsubscript{800} signal could be repeated.
five times without loss of amplitude, indicating that the effect was not simply due to oxidation of all the dithionite in the cuvette, but rather to the re-oxidation of a reduced acceptor, which we provisionally term ‘acceptor X’. We surmise that after recapping the cuvette, the excess dithionite reduced this acceptor as well as all the dissolved oxygen, resulting in loss of stable P$_{800}$ photobleaching. These findings establish that the reduction is reversible, which is key to performing a redox titration. Our working hypothesis is that the reduction of acceptor X results in a blockage of forward electron transfer from A$_0^-$. This results in rapid charge recombination of the P$_{800}^+A_0^-$ charge-separated state, which is undetectable as it is faster than the resolution of the spectrometer.

We estimated the midpoint potential of this acceptor by taking advantage of the fact that the solution potential of dithionite (i.e. the SO$_2^{-}$ ion) depends on the solution pH. Therefore, by varying the ambient reduction potential of the solution and by measuring the extent of P$_{800}$ photobleaching, the midpoint potential of acceptor X can be determined. To accomplish this, we used buffers at different pH values to modulate the reduction potential of the SO$_2^{-}$ ion and we measured the extent of P$_{800}$ photobleaching at each pH value. The potential of the dithionite solution was then independently measured in the presence of redox mediators (mediators
were not used in the photobleaching experiment, as they can act as rapid electron donors to $P_{800}^+$ and interfere with the measurement). As shown in Figure 2.1B, the apparent loss of $P_{800}$ photobleaching behaved as the reduction of a one-electron acceptor ($n = 0.99$) with a midpoint potential of $-502 \pm 3$ mV (v. SHE).

2.4.2 Ultra-fast pump-probe optical studies of charge recombination between $P_{800}^+$ and $A_0^-$

To test the hypothesis that the loss of $P_{800}$ photobleaching is due to rapid charge recombination between $P_{800}^+$ and $A_0^-$, we turned to ultra-fast pump-probe spectroscopy to observe the $P_{800}^+A_0^-$ charge-separated state directly. HbRCs in the presence of ascorbate (to re-reduce $P_{800}^+$ in any HbRCs from which the electron had escaped) or in the presence of dithionite at pH 10.0 (to reduce acceptor X) were excited with 100 fs laser pulses at 575 nm and absorption changes in the 620-850 nm region were probed for up to 3000 ps after the pump pulse. The decay of pump-induced spectral changes at all monitored wavelengths was analyzed by global fitting. The kinetics of the ascorbate-treated HbRCs could be described as the sum of three exponential decay components, while the dithionite-treated sample required only two components. The decay-associated spectra (DAS) are shown in panels A and B of Figure 2.2. The $\sim 25$ ps component in both samples is due to charge-separation, in agreement with our previous analysis.$^{10}$ (Note that
this component should include excitation energy equilibration, but we did not resolve this component from trapping, as it was not relevant to this study).

In the ascorbate-treated sample (Figure 2.2A), the ~650 ps component is due to forward electron transfer from \( A_0^- \); note that \( P_{800} \) remains oxidized under these conditions\(^{10}\). The non-decaying spectrum in this sample is primarily due to \( P_{800}^+ \), which persists for >1 ms. These two components are replaced by a slow decay component (~22 ns) in the dithionite-reduced sample (Figure 2.2B). Both \( A_0^- \) (670 nm) and \( P_{800}^+ \) (800 nm) contribute to this decay component, allowing us to assign it to charge recombination of \( P_{800}^+ A_0^- \). Because the rate was much longer than the timescale used, we examined the kinetics at 680 nm to follow \( A_0^- \) decay at a somewhat longer time scale (6000 ps) and were able to make a more accurate estimate of the decay time as ~15 ns. Although this estimate may not be an accurate measurement of the charge recombination lifetime due to the mismatch in timescales, it is in very good agreement with previously determined values for \( P_{800}^+ A_0^- \) charge recombination (Figure 2.2C)\(^9,34\). Thus, in the presence of dithionite, forward electron transfer from \( A_0^- \) is blocked and replaced by rapid \( P_{800}^+ A_0^- \) charge recombination. No other electron transfer events were observed.
2.4.3 Time-resolved EPR studies of $P_{800}^+$ in HbRCs reduced by Cr(II)EDTA

When HbRCs are frozen in the dark and continuously illuminated in the EPR cavity at 90 K, a symmetric EPR signal at $g \approx 2$ characteristic of a (B)Chl cation radical is observed (Figure 2.3, inset). This signal, which is assigned to $P_{800}^+$, almost completely disappears if the HbRCs have been previously reduced with the strong reductant Cr(II)EDTA ($E_m = -1$ V v. SHE)$^{35}$ prior to freezing. Unlike dithionite, which produces SO$_2$•$^-$ radicals that give rise to signals overlapping those of organic radicals$^{36}$, Cr(II)EDTA was used as a reductant in this experiment because it does not generate signals around $g \approx 2$. After thawing the Cr(II)EDTA-treated HbRCs in air and refreezing, the light-induced $P_{800}^+$ radical was restored. As shown above, charge recombination between $P_{900}^+$ and $A_0^-$ occurs in 15 to 20 ns at 300 K (and 50 to 60 ns at 5 K) in the presence of dithionite at pH 10.0, which is beyond the resolution of our EPR spectrometer in field modulation mode$^9$. Thus, disappearance of the light-induced $P_{800}^+$ radical at 345.6 mT can be used as a metric for the amount of acceptor X reduced.

If the magnetic field is held constant and the sample is flashed with a laser, the decay of the charge-separated state occurs with a lifetime of $\sim 2$ ms at 90 K, similar to what was observed in HbRCs from $H. chlorum$$^{37}$. Following reduction with Cr(II)EDTA, no flash-induced radical could be seen. After
exposing the sample to oxygen, a light-induced radical at 345.6 mT with the same magnitude and decay time was observed (Figure 2.3). Therefore, the behavior of the HbRC by optical spectroscopy at 298 K (see above) or by EPR spectroscopy at 90 K is similar, despite a modest change in lifetime. Further, the reduction of acceptor X is reversible, independent of the chemical nature of the reductant.

We estimated the reduction potential of this acceptor by measuring the loss of the light-induced \( g \approx 2 \) radical from \( P_{800}^+ \) following a single-turnover flash. When the frozen samples were subjected to a laser flash in the absence of a strong reductant, a transient \( g \approx 2 \) radical signal attributed to \( P_{800}^+ \) appears, followed by a decay with a lifetime of 1.6 ± 0.4 ms (Figure 2.4A). The amplitude of the flash-induced \( g \approx 2 \) EPR signal disappeared as the solution potential was lowered to –600 mV versus SHE prior to freezing. The lifetime of the radical species, moreover, did not change as the potential was lowered. The titration data are best fit using the Nernst equation with a midpoint potential of –496 mV ± 2 mV (v. SHE) and \( n = 0.99 \) (Figure 2.4B). The complete loss of the \( g \approx 2 P_{800}^+ \) radical signal following a single-turnover flash indicates that the endpoint of the titration is reached at the lowest attained solution potential.

2.4.4 EPR studies of the \( F_X \) cluster in HbRCs reduced by dithionite
We next tested the hypothesis that acceptor X in the HbRC is the interpolypeptide Fx iron-sulfur cluster. Iron-sulfur clusters have broad, low intensity charge-transfer bands in the visible spectrum, rendering optical spectroscopy less useful for direct observation of redox changes in Fx, particularly in a background of 22 highly absorbing chlorins. In contrast, the reduction of Fx as well as the oxidation of P800 can be readily measured by EPR spectroscopy, as their resonances are well separated (Fx is found at 140 mT and P800\(^+\) is found at 345.6 mT).

In order to use the low-field resonance at 140 mT as an indicator of the reduction state of the Fx cluster, we needed to confirm that it is natively in a \(S = 3/2\) ground spin state. We made use of heliobacterial membranes that had been washed in 100 mM sodium carbonate buffer (pH 11.5), which opens liposomes and allows removal of extrinsic proteins from both sides of the membrane\(^24\). These membranes still retain Cyt c\(_{553}\), which is covalently attached to a lipid, thereby allowing for rapid reduction of P\(_{800}\)\(^+\)\(^{38,39}\). Cyt c\(_{553}\) has been shown to reduce P\(_{800}\)\(^+\) in <1 ms at 298 K\(^{34}\), which is fast enough to outcompete the 15 ms charge recombination between P\(_{800}\)\(^+\) and Fx\(^-\)\(^8\) (see Figure 2.1A), and should therefore result in photoaccumulation of the P\(_{800}\) Fx\(^-\) state. When carbonate-washed membranes were incubated in the dark in the presence of dithionite at pH 6.0 to reduce Cyt c\(_{553}\), a P\(_{800}\) photobleaching
signal was seen on the first laser flash. However, it progressively disappeared with each subsequent flash, consistent with the finding that the acceptor X is not directly reduced by dithionite at pH 6.0 (Figure 2.1B), but rather is trapped in the reduced state through rapid reduction of P$_{800}^+$ by Cyt c$_{553}$. If such membranes were frozen immediately after the cessation of illumination, a resonance around 140 mT is observed. Membranes in the dark do not exhibit this signal (data not shown). Figure 2.5 (trace b) is a difference spectrum, in which the spectrum of membranes that had been treated with dithionite (pH 6.0) but left in the dark before freezing was subtracted from the spectrum of illuminated membranes. This procedure removes the signals of redox-active centers that are not light-induced. The resulting spectrum is similar to the previously reported spectrum of F$_X^-$ in isolated HbRCs, and we are therefore confident in assigning this signal to F$_X^{-8}$.

To determine the pH at which F$_X$ can be reduced chemically, HbRC cores were treated with sodium dithionite at pH 6.0 or pH 10.0 in the dark for several minutes before freezing. There was no signal at 140 mT at pH 6.0, and only a minor signal was present from contaminating octahedrally coordinated iron at $g \approx 4.3$ (not shown). However, at pH 10.0, where dithionite has a lower reduction potential, a strong signal was visible at 140 mT. When the spectrum at pH 6.0 was subtracted from the spectrum at pH
10.0, to remove the contaminating iron signal, the resulting difference spectrum (Figure 2.5, trace a) appears similar to that seen in the carbonate-washed membranes (Figure 2.5, trace b) and in the previously-reported light-induced signal of \( \text{F}_{X^-} \). The spectrum, which is the superposition of the parallel and perpendicular components of the \( g \)-tensor of the \( S = 3/2 \) system at 4 K, can be simulated with values of \( g_x = 2.08, g_y = 1.88, g_z = 1.98 \) and an E/D value of 0.02 (see Table 2.1 for all parameters). The lineshape, field position, and temperature dependence (data not shown) of this signal is characteristic of a fast relaxing [4Fe-4S]\(^{2+/1+}\) cluster in a \( S = 3/2 \) ground state, consistent with its identification as reduced \( \text{F}_{X^-} \). We did not observe any signals near \( g \approx 2 \) that were previously reported and attributed to \( \text{F}_{X-21} \), either in membranes or in HbRCs. These experiments clearly show that \( \text{F}_X \) in the HbRC can be reduced under conditions (i.e. dithionite at pH 10.0) that would result in the reduction of only a small fraction of the \( \text{F}_X \) cluster of PSI\(^{40}\), indicating that its reduction potential is higher.

### 2.4.5 Direct redox titration of the \( \text{F}_X \) cluster in HbRCs by EPR spectroscopy

To determine the midpoint potential of \( \text{F}_X \) directly, HbRCs buffered at pH 10.0 with 1 M glycine were titrated at room temperature by the addition of small aliquots of a concentrated solution of sodium dithionite (300 mM in 1 M glycine, pH 10.0) in the presence of a cocktail of electron mediators with
potentials ranging from -359 mV to -691 mV (v. SHE). After the solution potential stabilized, as determined by the measured voltage across the gold working electrode and the Ag/AgCl reference electrode (potential drift < 1 mV/min), the sample was removed and transferred to an EPR tube under anoxic conditions in dim green light and frozen in liquid nitrogen. This procedure was repeated until EPR samples poised at potentials from -350 mV to -600 mV (v. SHE) were obtained. These samples were stored in the dark in liquid nitrogen until measurement.

Figure 2.6A depicts the low-field resonances around 140 mT of the S = 3/2 ground spin state of Fx as a function of solution potential. The signal increased as the solution potential was lowered to -600 mV versus SHE (the resonance around 110 mT is likely from a small amount of contaminating cytochrome). Assuming that the titration endpoint is reached at this solution potential limit of sodium dithionite at pH 10.0, the data were freely fit using the Nernst equation with a midpoint potential of -517 ± 10 mV (v. SHE) and n = 0.65. Because we obtained free fits with n ≈ 1.0 in the optical and EPR titrations of light-induced P800+, we depict the data in Figure 2.6B with n fixed as 1.0. The near-identical redox value with the time-resolved optical measurement of P800 in Figure 2.1 and the EPR measurement of P800+ in Figure 2.3 indicates that the acceptor X reduced in these titrations is Fx.
Hence, the reduction potential of the F_X cluster in the HbRC is significantly more positive than that of F_X in PSI (ca. −705 mV measured in the presence of reduced F_A and F_B^{41, 42} and −610 mV measured in the absence of PsaC^{40, 43}).

2.4.6 Redox titration of the spin-polarized P_{800}^{+}F_{X}^{−} radical pair

The EPR signal in the ascorbic acid-reduced HbRCs produced by an actinic laser flash is derived from the spin-correlated P_{800}^{+}F_{X}^{−} radical pair. Van der Est and colleagues showed that this signal in membranes from H. chlorum is composed of both emissive (negative) and absorptive (positive) features with a slightly net absorptive polarization resulting from singlet-triplet mixing in the radical pair precursor state P_{800}^{+}A_0^{−}^{17}. We found an identical signal in HbRC samples from H. modesticaldum frozen in the absence of strong reductant, but it diminished as the potential was lowered to −600 mV (Figure 2.7A) by chemical titration with dithionite at pH 10.0 and a cocktail of electron mediators. As F_X is chemically reduced, light-induced electron transfer from A_0^{−} to F_X is blocked, leading to rapid charge recombination between P_{800}^{+} and A_0^{−}, the detection of which is beyond the resolution of the spectrometer. Thus, the amplitude of the spin-polarized P_{800}^{+}F_{X}^{−} radical pair observed is proportional to the fraction of reduced F_X clusters. The complete loss of the spin-polarized P_{800}^{+}F_{X}^{−} radical pair at −600 mV (Figure 2.7A) validates the previous assumption that the endpoint of the
redox titration of $F_X$ as measured by the $S = 3/2$ resonance at 140 mT (Figure 2.6) is reached with dithionite at pH 10.0. The data are best fit using the Nernst equation with a midpoint potential of $-501 \pm 4$ mV (v. SHE) and $n = 0.84$. We show the data with $n$ fixed as 1.0 in Figure 2.7B.

We found no evidence for the existence of a spin-polarized $P_{800}^{-}A_1^{-}$ radical pair under conditions of reduced $F_X$. Thus, we still have no experimental evidence for participation of a quinone in forward electron transfer in the HbRC comparable to that of $A_1$ in PSI$^{44}$. Although an intermediate acceptor may have remained undetected due to kinetics, as faster rates of oxidation than reduction may lead to an undetectable population, the presence of $F_X^{-}$ should have led to the detection of a spin-polarized $P_{800}^{-}A_1^{-}$ radical pair. One possibility is that the MQ might have been doubly reduced on addition of dithionite, and hence, remained undetected after reduction of $F_X$. A second possibility is that the reduction of $F_X$ prevents forward electron transfer from $A_0$ to an intermediate acceptor leading to rapid $P_{800}^{-}A_0^{-}$ charge recombination (see discussion). Nevertheless, in the absence of these contingencies, our results provide no reason to call into question the sequence of electron transfer in heliobacteria as $P_{800} \rightarrow A_0 \rightarrow F_X$.

Absorption detected magnetic resonance (ADMR) studies$^{45}$ have shown that the triplet state of $P_{800}$ ($^3P_{800}$) is formed in HbRCs under
conditions in which $F_X$ is reduced. However, we were unable to detect a spin-polarized EPR spectrum of this state. The lack of a spin-polarized EPR spectrum can be explained by the unique zero-field splitting parameters of $^3P_{800}$. Simulations show that with the D and E parameters determined in the ADMR studies, the intensity of the spin-polarized EPR spectrum of $^3P_{800}$ becomes extremely weak, making it undetectable by transient EPR (See Chapter 3).

2.4.7 Redox titrations of the PshBI, PshBII and HM1_2505 ferredoxins by EPR spectroscopy

The dicluster ferredoxins PshBI, PshBII and HM1_2505 from *H. modesticaldum* were heterologously expressed in *E. coli* and purified under strict anoxic conditions by Ni-affinity chromatography. Each ferredoxin was titrated using dithionite at pH 10.0 and a cocktail of electron mediators in the same manner as the titration of $F_X$. The EPR spectra of all three ferredoxins show distinct spectral differences between samples poised at potentials between $–400$ and $–475$ mV and those poised at $–600$ mV (Figure 2.8B, D, F). The spectra at the higher potentials show a predominantly rhombic lineshape consistent with the preferential reduction of only one $[4Fe-4S]^{2+/1+}$ cluster and are consistent with the published light-induced single-electron reduction spectra reported for PshBI and PshBII in HbRCS46, 47. On lowering the potential, the spectra change significantly, and at $–600$ mV the lineshapes
of PshBI and PshBII closely resemble the chemically reduced spin-spin interaction spectra reported previously (Figure 2.8B, C)\textsuperscript{46, 47}. The spectral changes observed between these potential ranges suggest the two [4Fe-4S]\textsuperscript{2+/1+} clusters have different midpoint potentials, as was first observed by Heinnickel et al. for PshBI\textsuperscript{23}. Accordingly, the titration data were fit with a modified form of the Nernst equation that assumes two redox centers with different midpoint potentials. The resulting curves were fit as two independent single electron processes with midpoint potentials of –480 ± 11 mV/–524 ± 13 mV (v. SHE) for PshBI, –453 ± 6 mV/–527 ± 6 mV (v. SHE) for PshBII and –452 ± 5 mV/–533 ± 8 mV (v. SHE) for HM1_2505 (Figure 2.8A, C, E). These values are in good agreement with the midpoint potentials measured for the two terminal ‘F\textsubscript{A} and F\textsubscript{B}’ clusters of the homodimeric reaction center from Chlorobium vibrioforme (–446 mV/–501 mV)\textsuperscript{33}.
2.5 Discussion

The thermodynamic midpoint potentials of the redox centers of an electron transfer chain are critical to understanding their function. Estimates of the midpoint potential of the HbRC terminal electron acceptor from earlier reports have ranged from −414 mV to −610 mV (v. SHE); the titration data has been fit as either a one-electron or two-electron process; and the cofactor titrated has been identified variously as a quinone or an iron-sulfur cluster \(^ {11,19,20,37,48,49}\). For example, a midpoint potential of −510 mV was measured by the flash-induced photobleaching at 590 nm in membranes of \textit{Heliobacterium chlorum} \((n = 1)\)\(^ {48}\), whereas a similar optical titration of \(P_{800}\) photobleaching measured at 800 nm for isolated RCs from \textit{Heliobacterium gestii} resulted in a midpoint potential of −442 to −444 mV in membranes \((n = 1)\) and −410 to −414 mV in isolated RCs \((n = 2)\)\(^ {20}\). Because of these uncertainties, we provisionally termed this acceptor ‘X’ and used four independent methods to determine its midpoint potential: (i) the decrease of stable \(P_{800}\) photobleaching by optical spectroscopy, (ii) the loss of the flash-induced \(g \approx 2\) radical from \(P_{800}^+\), (iii) the increase in the low-field resonance around 140 mT of the \(S = 3/2\) ground spin state of \(F_X\), and (iv) the loss of the flash-induced spin-correlated \(P_{800}^+F_X^-\) radical pair. The results of these
experiments indicate that the terminal acceptor X is indeed the F_X cluster and that its midpoint potential is \(-504 \pm 10\) mV \((n = 1)\).

We found no evidence for the participation of a quinone intermediate when F_X is reduced. Given that that there is no identified functional cofactor between A_0 and F_X at room temperature, the quasi-equilibrium thermodynamic model of electron transfer developed by Shinkarev and coworkers for purple bacterial RCs and PSI\(^{50,51}\) can be applied to estimate the midpoint potential of A_0 in the HbRC. In this model the lifetime of P\(_{800}^+\) \((k_{P800^+(obs)})\) can be expressed as a sum of exponential decay components for each cofactor involved in the regeneration of the ground state \((P_{800})\) following charge-separation, either via direct electron transfer (such as charge recombination from A_0\(^-\) to P\(_{800}^+\), \(k_{A0^-P800^+}\)) or via thermal repopulation of the excited singlet state P\(_{800}^*\) from the charge separated state P\(_{800}^+\)F_X\(^-\) followed by de-excitation (as is the case for P\(_{800}^*\) relaxation to P\(_{800}\), \(k_{P800^*P800}\)) weighted by the equilibrium constant between that cofactor \((A_0 \text{ and } P_{800}^*)\) and F_X \((\Delta G_{FXA0} \text{ and } \Delta G_{FXP800^*}\) respectively), the highest potential acceptor \((\text{Equation 1})\). Given that the charge recombination rates of the electron acceptors A_0 and F_X are known and the midpoint potential of F_X is now measured to be \(-504\) mV, it is possible to use this expression to estimate the midpoint potential of A_0.
Although the rate of de-excitation of the excited singlet state $P_{800}^*$ to the ground state $P_{800}$ is not known, for any reasonable lifetime the first term in the quasi-equilibrium model becomes insignificant ($0.1 \text{ sec}^{-1}$ or $0.0001 \text{ sec}^{-1}$ for $\tau_{PP} = 1 \text{ ps}$ or $\tau_{PP} = 1 \text{ ns}$, respectively) compared to the experimentally measured dark relaxation rate of $P_{800}^*$ ($k_{P_{800}^+ (obs)} = 1/(15 \text{ ms}) \approx 67 \text{ sec}^{-1}$). The midpoint potential of $A_0$ can then be calculated by solving for the term representing the difference in free energy between $F_X$ and $A_0$ ($\Delta G_{FXA0} = -350 \text{ meV}$). We therefore estimate the midpoint potential of the primary acceptor $A_0$ ($8^1$-OH Chl $a_F$) in the HbRC to be $-854 \text{ mV}$ at room temperature, a value $\sim 150 \text{ mV}$ more positive than its counterpart in PSI ($-1 \text{ V}$)\textsuperscript{52}.

Based on an amino acid sequence alignment between PsaA, PsaB and PshA (Figure 2.9), the Met serving as the axial ligand to the $\text{Mg}^{2+}$ of the $A_0$ Chl in PSI is predicted to be replaced by a Ser residue in PshA\textsuperscript{53}. It has been suggested that the unusual pair of a soft base (Met thioether sulfur) and soft acid ($\text{Mg}^{2+}$) may be responsible for the extremely low potential of the $A_0$ cofactor in PSI\textsuperscript{14}. The use of a hard base such as the Ser hydroxyl oxygen could explain the more positive potential of $A_0$ in the HbRC as predicted by the quasi-equilibrium model. Indeed, when the Met ligand to $A_0$ was altered to His in PSI, its midpoint potential became too oxidizing to pass the electron
forward to the $A_{1A}/A_{1B}$ quinones. A Ser residue should similarly lead to a higher midpoint potential for $A_0$, and in fact a change of the Met ligand to Ser in algal PSI resulted in a phenotype similar to the change to a His residue.

At temperatures below 150 K, the lifetime of $P_{800}^+$ is reduced from $\sim 15$ ms to $\sim 2$ ms. Although the titration of the light-induced $P_{800}^+$ EPR signal at 90 K tracks the reduction behavior of $F_X$, it does not unambiguously link the 2 ms charge recombination event to $P_{800}^+F_X^-$ recombination. The possibility that chemical reduction of $F_X$ prevents forward electron transfer from $A_0$ to another intermediate between $A_0$ and $F_X$, which recombines with $P_{800}^+$ in $\sim 2$ ms at cryogenic temperatures, cannot be excluded. Therefore, whether this reduction in the lifetime of $P_{800}^+$ is the result of charge recombination between $F_X^-$ and $P_{800}^+$ or an intermediate acceptor located between $A_0$ and $F_X$ and $P_{800}^+$ still remains unclear. Assuming an optimum in the Marcus curve (where reorganization energy matches the free energy difference between $A_0$ and $F_X$), application of the Moser-Dutton ‘ruler’ requires an edge-to-edge distance of $\sim 9$-10 Å to explain the 600-800 ps forward electron transfer rate from $A_0$ to $F_X$. This distance is 4-5 Å shorter than the distance between $A_0$ and $F_X$ in PSI. Whether the distance between $A_0$ and $F_X$ in the HbRC is in fact shorter than in PSI, or there is a yet
unidentified cofactor functioning as a bridge between A₀ and Fₓ, will likely require a high-resolution crystal structure of the HbRC.

The midpoint potential of Fₓ can be rationalized based on the need for both a sufficient Gibbs free energy drop to reduce acceptors (such as PshBI, PshBII and HM1_2505) and a sufficiently long P₈₀₀⁺Fₓ⁻ charge-separated lifetime for Cyt c₅₅₃ to reduce P₈₀₀⁺. **Figure 2.10** shows a plot of the lifetime of P₈₀₀⁺ as a function of the difference in Gibbs free energy between A₀ and Fₓ. We can imagine the consequence of a shift in the potential of Fₓ under two extremes. (i) If the midpoint potential of Fₓ were 50 mV more negative (~-550 mV), then ΔGₓA₀ would be only ~0.3 eV. While the Eₘ of ~-550 mV would provide greater driving force for electron transfer to the soluble ferredoxins, this would occur at the expense of the lifetime of the P₈₀₀⁺Fₓ⁻ charge-separated state. At 298 K, the lifetime of P₈₀₀⁺ would be reduced to ~1 ms, which is on the same order of magnitude as the forward electron transfer lifetime of Cyt c₅₅₃ to P₈₀₀⁺.³⁹,⁵⁷ In this case, recombination would begin to outcompete forward transfer from the cytochrome, thereby reducing the quantum yield of stable charge-separation. (ii) If the midpoint potential of Fₓ were shifted 50 mV more positive (~-450 mV), leading to ΔGₓA₀ ≈ -0.4 eV, the lifetime of P₈₀₀⁺ would be extended 10-fold, which would provide more time for successful binding and reduction of the soluble ferredoxins. However, the
longer-lived charge-separated state would come at the expense of a significant loss of driving force for the reduction of those ferredoxins, which are poised between −450 mV and −535 mV (Figure 2.8). These studies show that kinetics, as well as thermodynamics, specify the limits for the midpoint potentials of the electron transfer cofactors on the reducing side of the HbRC. Although the midpoint potential of F_X in the HbRC is ~100-200 mV more positive than the analogous F_X cluster in PSI (−610 mV\textsuperscript{40,43} to −705 mV\textsuperscript{41,42}) it is approximately equipotential with the F_A cluster (−530 mV\textsuperscript{58}), which is the cofactor with the highest redox potential on the acceptor side of PSI. This is an appropriate midpoint potential for the terminal acceptor of the HbRC (Figure 2.11), given the kinetic and thermodynamic constraints discussed above.
2.6 References


H. (2014) Evidence that histidine forms a coordination bond to the \( A_{0A} \) and \( A_{0B} \) chlorophylls and a second H-bond to the \( A_{1A} \) and \( A_{1B} \) phylloquinones in M688H\textsubscript{PsaA} and M668H\textsubscript{PsaB} variants of \textit{Synechocystis sp.} PCC 6803, \textit{Biochim. Biophys. Acta} 1837, 1362-1375.


2.7 Figures

Figure 2.1. Panel A: Kinetics of bleaching at 803 nm in isolated HbRCs in the presence of ascorbate or dithionite. “Air exposed” is the dithionite-treated sample after a brief inversion in the presence of air. Delay time is expressed on a log scale. Panel B: Redox titration of $P_{800}$ photobleaching. The $P_{800}$
photobleaching signal (in the ms timescale) was measured before and after a 1-hour treatment of 10 mM dithionite at pH ranging from 6.0 to 10.0. (The potential of each solution was measured separately in the presence of mediators.) Each point represents the average of at least three measurements. The data was fit to the Nernst equation with $E_m = -502$ mV (v. SHE) and $n = 0.99$. 
Figure 2.2. Ultra-fast pump-probe analysis of purified HbRCs. Purified HbRC particles in buffer (pH 10.0) containing either 10 mM ascorbate (and 20 mM PMS) or 10 mM dithionite were excited at 575 nm and probed in the red-NIR
region. The data in the 620-850 nm range were fit using global analysis with a sum of three exponentials for ascorbate-treated HbRCs (A) and two exponentials for dithionite-reduced HbRCs (B). The DAS of the kinetic components are shown in Panels A and B: the ~25 ps component (blue) of excitation trapping, the 660 ± 20 ps component of A₀⁻ decay (red, panel A), and the long-lived components corresponding to P₈₀₀⁺F⁻ (gray, A) or P₈₀₀⁺A₀⁻ (black, B). Panel C: Kinetics at 678 nm for HbRCs treated with 10 mM ascorbate and 20 mM PMS (black) or 10 mM dithionite (red).
Figure 2.3. Lifetime of the \( P_{800}^+ \) radical at 90 K after a laser flash measured under oxidizing and reducing conditions. The measurement was performed at a fixed magnetic field in as-isolated (solid line, \( \tau = 2.2 \) ms), Cr(II)EDTA reduced (short dashed line) and oxygen-exposed HbRCs (long dashed line, \( \tau = 2.6 \) ms). The oxygen exposed sample is the same one used for the Cr(II)EDTA reduction but was thawed and briefly mixed with air after
treatment with Cr(II)EDTA in the dark before refreezing. (Inset) Light-minus-dark EPR spectra at 90 K of the steady-state P800+ radical generated by continuous illumination in as-isolated (solid line), Cr(II)EDTA reduced (short dashed line) and oxygen exposed HbRCs (long dashed line). The arrow indicates the magnetic field position at which the transient P800+ radical lifetime measurements were made. EPR conditions: 25 µW microwave power, 9.34 GHz microwave frequency, 100 kHz modulation frequency and 0.1 mT modulation amplitude. The temperature was 90 K. For transient P800+ radical measurements using time-sweep mode data represent the average of 512 flashes using a conversion time of 0.32 ms and a static field position of 345.8 mT. The laser repetition rate was 0.5 Hz with a pulse width of 7 ns and an intensity of 20 mJ/pulse. The field position was determined by measuring the maximum amplitude of the steady-state P800+ radical generated by continuous illumination (0.1 W at 532 nm) before switching to time-sweep mode.
Figure 2.4. Redox titration of the flash-induced $P_{800}^+$ radical at $g \approx 2$ and 90 K. (a) Decay spectra of the $P_{800}^+$ radical at various solution potentials, and (b) amplitude of the transient $P_{800}^+$ radical as a function of solution potential. The redox titration of $F_X$ was fit with $E_m = -496 \pm 2$ mV and $n = 0.99$. EPR conditions are the same as in Figure 3. For each sample poised at a different solution potential the steady-state light-minus-dark $P_{800}^+$ spectrum was
measured using continuous illumination to determine the magnetic field position of the low-field peak of the radical. The EPR was then switched to time-sweep mode without removal of the sample and the magnetic field was fixed to this position. The kinetic traces are the average of 512 flashes of actinic light. Titration data in (b) plotted as a fraction of reduced Fx as a function of solution potential. The maximum amplitude of the transient light-induced P_{800}^{•} radical seen at solution potentials above –400 mV (v. SHE) was considered to represent 0% Fx reduction. The lack of any appreciable light-induced P_{800}^{•} radical at solution potentials of ~ –600 mV (v. SHE) indicated that the reduction of the acceptor was complete.
Figure 2.5. Difference spectrum (pH 10.0-minus-pH 6.0) of dithionite-reduced HbRCs (a) and light-minus-dark spectrum in carbonate-washed membranes (b). EPR conditions: 0.25 mW microwave power, 9.43 GHz microwave frequency, 1 mT modulation amplitude, and 100 kHz modulation frequency. The temperature was 4.3 K. Black traces are difference spectra and red traces are fittings. Parameters for the fitting of the EPR spectra are listed in Table 2.1.
Figure 2.6. Direct redox titration of the 140 mT resonance of the F$_X$– cluster with dithionite. (a) Representative low-temperature CW EPR spectra of the reduced F$_X$ cluster and (b) the corresponding titration curve fit with $E_m = -517 \pm 10$ mV and $n = 1$. EPR conditions: 2 mW microwave power, 9.43 GHz.
microwave frequency, 100 kHz modulation frequency and 2 mT modulation amplitude. The temperature was 4.3 K. All samples prepared under dim green light under strict anoxic conditions.
Figure 2.7. Redox titration of the flash-induced $P_{800}^{+}F_{X}^{-}$ spin-correlated radical pair with dithionite at 90 K. (a) Transient EPR spectra of the $P_{800}^{+}F_{X}^{-}$ radical pair at various solution potentials measured from the same samples used for Figure 2.4. (b) The corresponding titration curve fit with $E_m = -501 \pm 4$ mV and $n = 1$. The oxidative endpoint of the light-induced $P_{800}^{+}F_{X}^{-}$ spin
correlated radical pair were normalized to zero (equivalent to no reduction of F_X) and the reductive endpoint normalized to 1 as confirmed by the lack of any P_800^+F_X^- or light-induced P_800^+ signal at ~600 mV (see text).
Figure 2.8. Redox titrations and representative EPR spectra of heterologously expressed PshBI (A, B), PshBII (C, D) and HM1_2505 (E,F). Titration curves were fit with a modified form of the Nernst equation for two independent redox centers with different midpoint potentials (see Materials and Methods). The resulting fit for two single electron processes ($n = 1$) were $-480 \pm 11\text{ mV}/-524 \pm 13\text{ mV}$ (v. SHE) for PshBI, $-453 \pm 6\text{ mV}/-527 \pm 6\text{ mV}$ (v. SHE) for PshBII and $-452 \pm 5\text{ mV}/-533 \pm 8\text{ mV}$ (v. SHE) for HM1_2505. EPR
conditions: microwave power 10 mW, 9.34 GHz microwave frequency, 2 mT modulation amplitude and 100 kHz modulation frequency. The temperature was 15 K. The higher potential spectra (B,D,F, bottom) were multiplied either 3 or 6 fold to aid in comparison with respect to the lower potential spectra (B, D, F, top).
**Figure 2.9.** Amino acid alignment of PsaA and PsaB from *Thermosynechococcus elongatus* and PshA from *Heliobacterium modesticaldum*. Asterix symbol indicates residues involved in (PsaA/PsaB) or predicted to be involved in (PshA) interactions with the special pair (P$_{700}$/800) and A$_0$ electron transfer cofactors. The sequence alignment was carried out using ClustalW.
**Figure 2.10.** Plot of the $P_{800}^+$ lifetime at room temperature as a function of the difference in Gibbs free energy between $F_X$ and $A_0$ assuming a quasi-equilibrium model for charge recombination between $F_X^-$ and $P_{800}^+$. Using a 15 ms lifetime for $P_{800}^+$ and a 20 ns lifetime of $A_0^-$ when recombining with $P_{800}^+$, the free energy gap between $F_X$ and $A_0$ is predicted to be $-350$ meV (dashed line). The effect of shifting the midpoint potential of $F_X$ 50 mV higher (green) or lower (red) than the experimentally determined value of $-504$ mV alters the lifetime of $P_{800}^+$ from $\sim 1$ ms at a midpoint potential of $-550$ mV to $\sim 110$ ms at a midpoint potential of $-450$ mV.
Figure 2.11. A comparison of the midpoint potentials and kinetics of the electron transfer cofactors in Photosystem I (PSI) (left) and the heliobacterial reaction center (HbRC) (right).
### 2.8 Tables

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Isolated HbRC</th>
<th>Carbonate-washed membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_x$</td>
<td>2.08</td>
<td>2.05</td>
</tr>
<tr>
<td>$g_y$</td>
<td>1.88</td>
<td>2.00</td>
</tr>
<tr>
<td>$g_z$</td>
<td>1.98</td>
<td>1.97</td>
</tr>
<tr>
<td>$\Delta B_x$ (mT)</td>
<td>81</td>
<td>79</td>
</tr>
<tr>
<td>$\Delta B_y$ (mT)</td>
<td>109</td>
<td>89</td>
</tr>
<tr>
<td>$\Delta B_z$ (mT)</td>
<td>26</td>
<td>11</td>
</tr>
<tr>
<td>D (MHz)</td>
<td>$&gt;10^5$</td>
<td>$&gt;10^5$</td>
</tr>
<tr>
<td>E/D</td>
<td>0.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Table 2.1.** EPR fitting parameters used to simulate the $F_X^-$ spectrum at $X$-band. See [Figure 2.5](#).
Chapter 3

Electron Transfer Processes in Homodimeric Type I Reaction Centers from Heliobacteria, Green Sulfur Bacteria, and Chloracidobacteria

Published in part as:
3.1 Abstract

In photosynthetic reaction centers (RCs), reduction of the secondary acceptors leads to triplet charge recombination of the primary RP. This process is spin selective and populates only the T$_0$ state of the donor triplet state. As a result, the triplet state of the donor has a distinctive spin polarization pattern that can be measured by transient electron paramagnetic resonance (trEPR) spectroscopy. In heliobacterial reaction centers (HbRCs), the primary donor, P$_{800}$, is composed of two bacteriochlorophyll $g'$ molecules and its triplet state has not been studied as extensively as those of other reaction centers. Here, we present trEPR and optically detected magnetic resonance (ODMR) data of $^{3}$P$_{800}$ and show that although it can be detected by ODMR it is not observed in the trEPR data. We demonstrate that the absence of the trEPR spectrum is a result of the fact that the zero-field splitting tensor is maximally rhombic, which results in complete cancellation of the absorptive and emissive polarization in randomly oriented samples. However, in partially oriented samples, the cancellation is predicted to be incomplete making the trEPR spectrum possible to detect. Under non-reducing conditions, trEPR can be used to track formation of the P$^*$F$_X^-$ state as well as estimate the lifetime of the forward electron transfer (ET) event from A$_0^-$ to F$_X$. We present here the first trEPR
characterization of chlorosome-depleted \textit{C. thermophilum} membranes that contain an oxygen stable homodimeric Type I RC (CabRC). Under non-reducing conditions a RP signal similar to the P$^*$F$\lambda$ RP in HbRCs and green sulfur bacterial RCs (GSbRCs) is observed. Under reducing conditions, the RP is replaced with a broad triplet spectrum characteristic of charge recombination to the primary donor, a dimer of Zn-BChl a'/Zn-BChl a' (P$_{840}$). When the RP is titrated chemically, the potential of the F$_\lambda$ cluster is found to be poised at -581 ± 7 mV v. SHE. Time-resolved optical spectroscopy at 298 K and time-resolved field-modulated continuous-wave (CW) EPR at 90 K confirm long-lived charge-separation is achieved in CabRCs with similar kinetics as HbRCs and GSbRCs. Retention of a slow charge recombination event, assigned to P$_{840}^*$F$_B^-$, following treatment with 500 mM NaCl suggests the PscB protein is more tightly bound to the RC than either PshBI/PshBII in heliobacteria or PscB in green sulfur bacteria (GSB).

3.2 Introduction

All known photosynthetic RCs share a common structural motif with two branches of ET cofactors extending across the complex from a pair of chlorophyll molecules, which act as an electron donor. The pair of chlorophylls is referred to as P$_\lambda$, where $\lambda$ is the wavelength of their absorbance maximum. In Type I RCs the two branches converge at an
interpolypeptide $[4\text{Fe-4S}]^{2+/1+}$ cluster, $F_X$. Absorption of light leads to ET along the chain of acceptors and generation of the stable charge separated state $P^+F_X^-$. In the absence of an electron acceptor, this state decays via charge-recombination with $P^+$ in $\sim 15$ ms at room temperature\textsuperscript{1, 2}. In the homodimeric Type I RCs of heliobacteria and GSB, small extrinsic oxygen labile bacterial-type ferredoxins harboring two additional $[4\text{Fe-4S}]^{2+/1+}$ clusters ($F_A$ and $F_B$) extend the ET chain past $F_X$. In heliobacteria two different ferredoxins (PshBI and PshBII) function as equivalent electron acceptors and readily dissociate at low ionic strength (100 mM NaCl)\textsuperscript{3}. In GSbRCs, the PscB protein is bound more tightly and serves instead as an intermediate ET component that promotes reduction of soluble bacterial-type ferredoxins\textsuperscript{4}. Like PshBI and PshBII in heliobacteria, PscB can also be removed from the RC core, however conditions of moderate ionic strength are required (500 mM NaCl)\textsuperscript{5}. In the presence of PshBI or PscB the charge-recombination lifetime of $P^+$ in HbRCs and GSbRCs is increased to $\sim 150$ ms\textsuperscript{2, 6, 7}. In contrast, no kinetic data exists for the oxygen stable CabRC\textsuperscript{8, 9}.

When the ET chain is intact, trEPR can be used to characterize the series of light-induced radical pairs (RPs) formed during ET. Van der Est et al. originally assigned the observed RP signal in HbRCs and GSbRCs to the $P^+$ contribution of the final $P^+F_X^-$ charge separated state\textsuperscript{10}. In both the HbRC and
GSbRC, a net absorptive polarization is observed which can be simulated by including in the calculation singlet-triplet mixing during a RP precursor state with a lifetime of \( \sim 700 \text{ ps} \). These results are consistent with the optically measured lifetime of \( A_0^- \) observed in both HbRCs and GSbRCs, supporting the hypothesis that ET proceeds as \( P_{800/840} \rightarrow A_0 \rightarrow F_X \).  

If the secondary acceptor \( F_X \) in Type I RCs or \( Q_A \) in Type II RCs is chemically reduced, removed, or otherwise inactivated, charge recombination occurs. The spin selectivity of this charge recombination reaction has been studied extensively and is dominated by triplet recombination in all cases. The strongest evidence for the triplet pathway is the characteristic spin-polarized trEPR spectrum of the donor. This spectrum was first observed by Dutton, Leigh and co-workers in purple bacteria. Thurnauer, Katz and Norris showed that the polarization pattern \( A/E/E/A/A/E \) (\( A = \) absorption, \( E = \) emission) occurs as a result of singlet-triplet mixing in the primary RP. In the strong magnetic field of an EPR spectrometer, the spin-spin coupling in the RP is much weaker than the Zeeman interaction and the singlet state is mixed only with the \( T_0 \) triplet sublevel. As a result, only the \( T_0 \) sublevel of the donor triplet state is populated by recombination. In contrast, when a triplet state is generated by intersystem crossing (ISC), the population of the sublevels is determined by
how strongly each of them is perturbed by spin-orbit coupling. This perturbation depends on the orientation of the molecule in the magnetic field and hence for some orientations, selective population of the $T_0$ sublevel occurs but for others $T_\uparrow$ and $T_\downarrow$ are populated $^{27,32}$.

Of the triplet states in Type I RCs, that of the P$_{700}$ in the heterodimeric RC Photosystem I (PSI) has been studied most extensively$^{22,25,29,33}$. The ZFS parameters of $^3$P$_{700}$ at 4 K are the same as those of isolated Chl $\alpha$, indicating that the triplet excitation is localized on a single chlorophyll molecule$^{22,25}$. At room temperature, however, the ZFS parameter, $E$, becomes smaller. This change can be explained as a result of incoherent hopping of the excitation between the two chlorophylls of P$_{700}$$^{29}$. In GSB, the donor triplet state ($^3$P$_{840}$) shows similar behavior to the purple bacteria with ZFS parameters consistent with delocalization over the special pair$^{23,34,35}$. In contrast, the triplet states of P$_{840}$ in CabRCs and P$_{800}$ in HbRCs have not been as well studied (in the case of CabRCs no published characterization exists). There are two early reports of the steady-state EPR spectra of triplet states in HbRCs and a later, more extensive study using absorption-detected magnetic resonance (ADMR)$^{36-38}$. In both of the EPR studies, only the two outermost features of the triplet spectrum were observed. It was suggested that these features contained two components with different $D$ and $E$ values, which
were ascribed to $^3P_{800}$ and antenna triplets on the basis of their dependence on the reduction state of the acceptors. The $D$ and $E$ values obtained from the spectrum are in agreement with those observed by ADMR. However, the reported polarization pattern (A/E) of the spectrum is the same for both components, which is not consistent with two different mechanisms (ISC and charge recombination) for their formation. Moreover, the pattern is opposite to that reported more recently for antenna triplet states in HbRCs under oxidizing conditions. These discrepancies, as well as the lack of kinetic or thermodynamic information for the CabRC, led us to re-investigate the spin-polarized EPR spectra and charge recombination kinetics of all homodimeric Type I RCs (HbRCs, GSbRCs and CabRCs) under oxidizing and reducing conditions.

Under reducing conditions, the characteristic A/E/E/A/A/E polarization pattern of a triplet state formed by charge recombination is observed for GSbRCs and CabRCs but not for HbRCs. This difference is explained as a result of differences in the ZFS parameters of BChl $a$ and BChl $g$. We will show that when $D = 3E$, the intensity of the polarization pattern of the charge recombination triplet becomes zero because of cancellation of the absorptive and emissive contributions. This means that for solution samples of HbRC, the charge-recombination triplet cannot be observed and hence
optical methods or oriented samples must be used to demonstrate whether it is present or not.

Under non-reducing conditions, CabRCs display biphasic charge recombination kinetics at room temperature with lifetimes of 20-30 ms and 150-300 ms consistent with charge recombination events from F_X^- and F_B^- in HbRCs and GSbRCs\(^{1,2,6,7}\). The slow 150-300 ms phase is retained following treatment with 500 mM NaCl but is reduced upon prolonged exposure with sodium thiocyanate (NaSCN). However, under these conditions the extent of total charge-separation is also significantly reduced, most likely as a result of concomitant damage to F_X. Reversible charge-separation is also observed at cryogenic temperatures. At 90 K, the decay of P_{840}^+ is monophasic and has a lifetime of 82 ms. In addition, a net absorptive spin-polarized RP similar to P_{840}^+F_X^- in GSbRCs and P_{800}^+F_X^- in HbRCs is observed by trEPR. Therefore, the amplitude of the light-induced RP can be used as an indirect marker for the reduction state of the F_X. The loss of the RP signal as a function of the solution potential follows a theoretical one-electron Nernst curve with a midpoint potential of \(-581 \pm 7\) mV (v. SHE).

3.3 Materials and Methods

3.3.1 Preparation and purification of heliobacterial reaction centers
HbRCs were isolated as previously described by Heinnickel et al. All manipulations were carried out under strict anoxic conditions under dim green light. For trEPR measurements, samples were prepared by incubating HbRCs at a BChl $g$ concentration of 0.9 mM in 50 mM MOPS pH 7.0 with 0.02% dodecyl maltoside (DDM), 10% glycerol and 50 mM sodium ascorbate for 10 minutes in the dark for the oxidized sample or 50 mM sodium dithionite at pH 10.0 for 10 minutes in the dark for the reduced sample, prior to freezing in liquid nitrogen. BChl $g$ concentrations were calculated using the absorbance at 788 nm and an extinction coefficient for BChl $g$ of 110 mM$^{-1}$ cm$^{-1}$.

3.3.2 Preparation and purification of chlorosome-depleted membranes from C. tepidum

Chlorosome-depleted membranes were isolated from 2 L of Chlorobium tepidum cells grown to stationary phase as described in Klughammer et al. with the exception that 10 mM potassium phosphate buffer at pH 7.2 with 150 mM NaCl was used to resuspend the whole cells and chlorosome-depleted membranes. All manipulations were carried out under strict anoxic conditions under dim green light. For trEPR measurements, samples were prepared by incubating chlorosome-depleted membranes (OD$_{600nm}$ = 3.7) with 50 μM PMS and 50 mM sodium ascorbate for
10 minutes in the dark for the oxidized sample or with 50 mM sodium dithionite at pH 10.0 for 10 minutes in the dark for the reduced sample prior to freezing in liquid nitrogen.

3.3.3 Preparation and purification of chlorosome-depleted membranes from *C. thermophilum*

*Chloracidobacterium thermophilum* was grown and chlorosome-depleted membranes isolated by Dr. Marcus Tank in Dr. Bryant's laboratory at Penn State. The protocol for growth and membrane isolation was similar to the one described by Tsukatani et al. with the exception that 10 mM potassium phosphate buffer at pH 7.2 with 150 mM NaCl was used to resuspend the *C. thermophilum* whole cells and chlorosome-depleted membranes (no NaSCN was used unless specified in the text)\(^2\). For trEPR as well as field-modulated time-resolved CW EPR measurements, samples were prepared by incubating chlorosome-depleted membranes (OD\(_{810\text{ nm}} = 2.0\)) with 30 mM sodium ascorbate for 10 minutes in the dark for oxidized samples and 50 mM dithionite at pH 10.0 in the dark for reduced samples prior to freezing. For optical measurements chlorosome-depleted membranes (OD\(_{810\text{ nm}} \approx 0.6\)) were incubated with 30 mM sodium ascorbate under the solution conditions described below (see section 3.3.4).

3.3.4 Time-resolved optical spectroscopy
Time-resolved absorbance changes at 840 nm were measured using a commercial JTS-10 pump-probe spectrometer with a path length of 1 cm (Biologic LLC) adapted with a pulsed Nd:YAG actinic laser with a pulse width of ~400 ns and a single pulse energy of 3.0 mJ/pulse at 532 nm (Clark MXR ORC-1000). Detection light was provided by the built-in, pulsed, 810 nm LED diode filtered at 840 nm using an interference filter (FWHM = 10 nm)(Edmund Optics). Data represent the average of three light-minus-dark single-turnover events. The dark data set was collected following each actinic collection by physically blocking the actinic light from hitting the sample while still firing the laser. This was done to subtract an electrical artifact generated by the laser’s Q-switch operation. Data collection begins 300 μsec following the actinic flash due to interference from stray actinic laser light hitting the detector. Decay kinetics at 840 nm were fit using a nonlinear least squares multi-exponential fitting algorithm in Igor Pro.

To probe the binding strength of PscB to the CabRC, the sensitivity of the slow (150-300 ms) kinetic phase to ionic strength and chaotrope treatment were tested using anoxic buffers and measurement conditions (See Figure 3.10). The sacrificial electron donor sodium ascorbate was maintained at 30 mM throughout. Following time-resolved measurement of the charge recombination kinetics of P$_{840}^+$ in chlorosome-depleted
membranes under anoxic conditions (See Figure 3.10A), the sample was pelleted by ultracentrifugation at 207,000 x g for 30 minutes at 4 °C and the pelleted membranes resuspended in equal volume of 100 mM sodium carbonate buffer at pH 11.5 with 500 mM NaCl. Sodium carbonate has been shown to open liposome vesicles connecting the intraliposomal volume with the bulk solution thereby preventing soluble proteins from being trapped inside membrane vesicles. After 30 minutes of incubation in the sodium carbonate/NaCl buffer at 4 °C, membranes were again pelleted via ultracentrifugation as described above. The supernatant was decanted, the resulting membrane fraction resuspended with an equal volume of purification buffer (10 mM potassium phosphate, 150 mM NaCl), and the time-resolved charge recombination kinetics measured again (See Figure 3.10B). Membranes were again collected via ultracentrifugation and were resuspended with an equal volume of buffer containing 10 mM potassium phosphate pH 7.2, 150 mM NaCl and 1 M NaSCN. After 30 minutes (See Figure 3.10C) or 60 minutes (See Figure 3.10D) of exposure to the NaSCN-containing buffer, measurement of the charge recombination lifetime of P840* was repeated.

3.3.5 Redox titration of the P840*FxF RP from CabRC
The room temperature redox titration of chlorosome-depleted membranes of *C. thermophilum* was carried out as described in Ferlez et al.\(^4\). Briefly, an anoxic master mix of chlorosome-depleted membranes (OD\(_{810\text{ nm}}\) ≈ 1.0) and electron mediators (methyl viologen, benzyl viologen, dimethyl triquat and triquat) in 100 mM glycine buffer at pH 10.0 were poised at solution potentials ranging from –283 mV to –592 mV v. SHE by adding small aliquots of concentrated sodium dithionite (500 mM) in 1 M glycine buffer pH 10.0 or 100 mM potassium ferricyanide in water. The solution potential was measured using a high-impedance digital voltage meter connecting a gold working electrode and a Ag/AgCl reference electrode (Microelectrodes Inc) that was previously calibrated using a saturated solution of quinhydrone. After addition of dithionite or ferricyanide, the solution potential was allowed to stabilize for 5-10 minutes until the potential no longer changed. At this point 120 μL of the master mix was removed and rapidly transferred to an EPR tube and flash frozen in liquid nitrogen. This procedure was repeated until samples spanning the potential range of –283 to –592 mV were prepared. Samples were stored in liquid nitrogen until trEPR analysis. Titration data was fit using the Nernst equation and a nonlinear least squares fitting algorithm in Igor Pro (Igor Pro, Lake Oswego, OR).
3.3.6 Transient and field-modulated time-resolved continuous-wave EPR spectroscopy

trEPR measurements were made using a modified Bruker E300 EPR spectrometer at X-band in direct detection mode. A Flexline dielectric resonator (ER 4118 X-MD-5W1, Bruker BioSpin Corp., Billerica, MA) was used and the temperature was maintained at 90 K with an ER 4118CF liquid nitrogen cryostat (Bruker BioSpin Corp., Billerica, MA) and an ITC-4 controller. The transient EPR signal was collected with a home-built broadband amplifier (bandwidth >500 MHz), and digitized using a 500 MHz bandwidth, 8-bit, 2GS/s, Model CS85G PCI card (DynamicSignals LLC, Lockport, IL). The software for controlling the spectrometer and for data acquisition was written in-house in LabView. Three dimensional time/field/amplitude data sets were collected following single-turnover flashes at 20 mJ per pulse from a Vibrant 355Ii laser (Opotek, Inc., Carlsbad, CA) running at 10 Hz and a wavelength of 532 nm with a pulse width of 7 ns. Each time/field data set consists of 128 transient EPR signals collected and averaged at each fixed magnetic field at a resolution of 0.5 mT for triplet spectra and 25 μT for RP spectra. Boxcar spectra were extracted from the field/time data sets by integrating the EPR signal between 1-2 μsec following the laser flash.
For measurement of the \( P_{840} \) lifetime in \textit{C. thermophilum} chlorosome-depleted membranes at 90 K, the trEPR spectrometer described above was used with field-modulation (100 kHz) and data collection carried out using the built-in detector in the bridge operating in time-sweep mode. The magnetic field was fixed at the low-field peak (344.9 mT) and high-field trough (345.7 mT) of the light-induced radical at \( g \approx 2.0 \) and the sample was interrogated with pulses of actinic light at 532 nm and 20 ml/pulse at a repetition rate of 0.5 Hz using the same actinic laser used for the trEPR experiments described above. Data represent the average of 8,192 actinic flashes and were fit using a nonlinear least squares multi-exponential fitting algorithm in Igor Pro (Igor Pro, Lake Oswego, OR).

\subsection*{3.3.7 ODMR spectroscopy}

The principle of the ODMR technique, reviewed by D. Carobnera, will be briefly summarized here\textsuperscript{45}. ODMR is a double resonance technique based on the principle that, when a steady-state population of a triplet state is generated under continuous illumination, the application of a resonant microwave electromagnetic field between a pair of spin sublevels of the triplet state, generally induces a change of the steady-state population of the triplet state, due to the anisotropy of the decay and population rates of the spin sublevels. The induced change of the triplet population may be detected
as a corresponding change of the emission and/or absorption of the system. In particular, absorbance detected magnetic resonance (ADMR) detects the microwave-induced change in the steady state absorption of the chromophore carrying the triplet state, whereas fluorescence detected magnetic resonance (FDMR) detects the changes in the emission.

FDMR and ADMR experiments were performed using a home-built apparatus. Amplitude modulation of the applied microwave field is used to greatly increase the signal to noise ratio by means of a phase sensitive lock-in amplifier (EG&G 5220). In the FDMR experiments the fluorescence, excited by a halogen lamp (250W) focused into the sample and filtered by a broadband 5 cm solution of CuSO$_4$ 1 M, was collected at 45 degrees through a 780 nm cut-on and appropriate band-pass filters (10 nm FWHM) by a photodiode. The photodiode signal was demodulated after entering the lock-in amplifier. In ADMR, the same excitation lamp was used but without filters before the sample, except for 5 cm water and heat filters. The beam was focused into the monochromator after passing the sample and finally collected by the photodiode. The amplitude modulation frequency was 33 Hz and the microwave power 500 mW.

By fixing the microwave frequency at a resonant value and sweeping the detection wavelength, microwave-induced Triplet-minus-Singlet (T-S)
spectra were collected. All of the ODMR experiments were performed at 1.8 K. At this temperature spin-lattice relaxation is inhibited and the ODMR signal is detectable. The samples were diluted to give a final OD 788 nm = 1.0 with a 1 mm light path (BChl g concentration of 91 μM). Degassed glycerol was added to the samples to a final concentration of 60% v/v, in order to obtain homogeneous and transparent matrices upon freezing. Reduced samples were prepared by adding a few microliters of a freshly prepared 500 mM sodium dithionite solution, to reach a final concentration of 50 mM. After five minutes of dark incubation, the samples were inserted into the cryostat at a temperature of 270 K and subsequently cooled to 1.8 K under illumination.

3.4 Results

3.4.1 Charge recombination $^3P_{800}$ in HbRCs

A diagram showing the ET kinetics and pathways in HbRCs under oxidizing (left) and reducing (right) conditions is presented in Figure 3.1. In both cases, excitation of $P_{800}$ results in rapid ET to $A_0$ ($\tau \approx 25$ ps)\textsuperscript{12}. Under oxidizing conditions secondary ET to $F_X$ then occurs with a lifetime of $\sim 700$ ps and at low-temperature charge recombination to the ground state has a lifetime of 2-4 ms\textsuperscript{39, 48}. If HbRC samples are chemically reduced prior to excitation (Figure 3.1, right), ET past $A_0$ is blocked because $F_X$ has been
chemically reduced\textsuperscript{44}. In this case, triplet recombination occurs and the triplet state of the donor relaxes to the ground state with a lifetime of \(~500\) \(\mu\text{sec}\) as measured optically at \(5\) \(\text{K}\)\textsuperscript{48,49}. These results as well as the ADMR results of Vrieze et al. suggest that an \(A/E/E/A/A/E\) charge recombination triplet should be observable with trEPR. To determine if such a signal was present following reduction of \(F_X\) we examined the redox behavior of the trEPR spectra for GSbRCs and HbRCs.

3.4.2 trEPR spectra of HbRCs and GSbRCs under oxidizing and reducing conditions

Figure 3.2 shows a comparison of trEPR spectra of GSbRCs and HbRCs obtained under oxidizing and strongly reducing conditions at 90 K. GSbRCs and HbRCs are both homodimeric type I RCs and their ET kinetics are essentially the same\textsuperscript{34,50}. However, in HbRCs the primary donor is composed of two BChl \(g'\) molecules, while in GSbRCs the donor is a BChl \(a'\) dimer. Under oxidizing conditions (spectra a and d) both types of RCs give trEPR spectra with a sharp emission/absorption (E/A) polarization pattern from the \(P^+F_X^-\) RP and broad features on either side of the RP spectrum due to chlorophyll triplet states formed by ISC. The shape of the triplet contribution is different for the two types of RCs because HbRCs contain BChl \(g\), while GSbRCs contain BChl \(a\).
When F_X is chemically reduced prior to freezing with sodium dithionite at pH 10.0 (Figure 3.2b and 3.2e), the contribution from P^*F_X^- disappears. In GSbRCs (Figure 3.2b) this is accompanied by the appearance of a triplet spectrum with the A/E/E/A/E polarization pattern characteristic of selective population of the T_0 state by charge recombination\textsuperscript{23}. In contrast, in HbRCs (Figure 3.2e) the loss of P^*F_X^- is not accompanied by any change in the triplet spectrum. This can be seen most clearly in the reduced-minus-oxidized difference spectra in Figures 3.2c and 2f.

The red curves in Figure 3.2 are simulations of the triplet component based on the zero-field splitting parameters given in Table 3.1. For GSbRCs, the D and E values of 3P_{g40} obtained by fitting the triplet component in Figure 3.2c are in good agreement with those reported previously from trEPR and ODMR measurements\textsuperscript{23,51}. Under oxidizing conditions, the triplet component of the GSbRC spectrum (Figure 3.2a) can be simulated as the sum of a triplet formed by ISC and a small contribution from 3P_{g40} in a ratio of 91:9. The minor contribution from 3P_{g40} is from a fraction of RCs in which ET past A_0 is blocked, probably because of damage to F_X during the isolation procedure. The D value of the antenna triplet is larger than that of BChl a and the line width is large suggesting that this component is due to several different
triplet species (chlorophylls and carotenoids) in a distribution of environments. In contrast to GSbRCs, the triplet spectra observed for HbRCs contain only a contribution from \(^3\text{BChl g}\) formed by ISC in the antenna and no contribution from \(^3\text{P}_{800}\) is observed. The spectrum can be simulated using the D and E values of the two antenna triplets obtained from ODMR experiments, which will be discussed below.

3.4.3 ODMR detection of \(^3\text{P}_{800}\)

Because no contribution from \(^3\text{P}_{800}\) was observed in the trEPR spectrum of HbRCs under reducing conditions, we performed ODMR experiments under similar conditions to determine whether \(^3\text{P}_{800}\) is being formed. Figure 3.3 shows a comparison of the fluorescence detected magnetic resonance (FDMR) spectra of HbRCs frozen under oxidizing and reducing conditions (green and black spectra, respectively). The spectra are plots of the change in the fluorescence at \(\lambda > 780\) nm as a function of the applied microwave frequency. In this wavelength range only fluorescence from BChl \(g\) is observed and the change in fluorescence occurs when the microwave frequency comes into resonance with a pair of triplet sublevels split by the zero-field splitting. In general, peaks are observed at \(|D|+|E|\), \(|D|–|E|\) and \(2|E|\). The peaks between 880-1000 MHz are the \(|D|+|E|\) transitions from BChl \(g\) molecules in different environments. The peaks near 450 MHz
are the corresponding $|D|-|E|$ and $2|E|$ transitions. For BChl $g$, $|D| \approx 3|E|$, which means the $2|E|$ and $|D|-|E|$ transitions occur at approximately the same frequency. However as discussed by Vrieze et al., the contribution from the $2|E|$ transition is weak because decay and population rates of the $x$- and $y$-sublevels of the triplet are similar$^{38}$.

Figure 3.4 shows a reconstruction of the FDMR spectra of the reduced sample by a sum of three Gaussian components corresponding to three different types of BChl $g$. As can be seen in Figure 3.3, the peak at 980 MHz is not present in the oxidized sample but appears when $F_X$ is reduced. Careful comparison of the FDMR spectra and their detection wavelength dependence (not shown) reveal that there is also a peak at 475 MHz that appears only when $F_X$ is reduced. The peaks at 980 MHz and 475 MHz agree well with the position of the $|D|+|E|$ peak of $^3P_{800}$ in ADMR reported previously$^{38}$. The FDMR spectra measured with different detection wavelengths and microwave-induced triplet-minus singlet absorption difference spectra collected at different microwave frequencies (not shown) reveal three different types of BChl $g$ molecules with different $Q_y$ absorbance maxima and $|D|$ and $|E|$ values. As shown in Figure 3.4, the FDMR spectrum can be decomposed into three components corresponding to these three different types of BChl $g$. The values of the ZFS parameters and absorbance

131
maxima are summarized in Table 3.2 and are in very good agreement with previously reported values. Importantly the values of D and E obtained from the FDMR spectrum of the oxidized sample, reproduce the trEPR spectra of both the oxidized and reduced HbRC samples as can be seen in Figure 3.2d and e.

3.4.4 Simulation of triplet state polarization as a function of the zero-field splitting parameters D and E

Our ODMR data confirm that under reducing conditions $^3P_{800}$ is formed. However, in the trEPR data there is no evidence of a spin-polarization pattern from this species. To investigate this further, we have calculated the expected spin-polarization pattern for $^3P_{800}$. From Tables 3.1 and 3.2 we see that an important difference in the ZFS parameters of the triplet states of the two donors is that $^3P_{800}$ has a much larger E-value because it is composed of BChl $g'$ while P$_{840}$ is BChl $a'$. Thus, we have calculated a series of chlorophyll triplet spectra for different values of E by RP recombination (Figure 3.5, right) as well as ISC (Figure 3.5, left). For the RP recombination triplet, the polarization pattern is A/E/E/A/A/E, while for ISC an E/E/E/A/A/A pattern is obtained. With E = 0, the ZFS tensor is axial and the splitting is the same for molecules oriented with any axis in the $xy$ plane oriented along the magnetic field. This results in the strong inner peaks in both the RP recombination and ISC triplet spectra. As the value of E
increases, the rhombicity of the ZFS tensor increases and the features from
the x- and y-orientations no longer appear at the same position in the
spectrum. The maximum rhombicity is attained when E = D/3. Under these
conditions the splitting for the x-orientation is zero and is equal and opposite
for the y- and z-orientations. For the RP recombination triplet spectra on the
right of Figure 3.5, it can be seen that the intensity of the spectrum
decreases as E increases and with E = D/3 no spectrum is obtained. For 3P800,
the ODMR data show that E = 84.2×10^{-4} \text{ cm}^{-1} \text{ and } D/3 = 80.9×10^{-4} \text{ cm}^{-1}.
Hence, the intensity of the spin-polarized trEPR spectrum of 3P800 should be
very close to zero.

To illustrate why the intensity of the RP recombination triplet is zero
when D/E = 3, a comparison of the calculated spectra of 3P940 in GSbRCs and
3P800 in HbRCs is shown in Figure 3.6. In the top part of the figure,
contributions of the absorptive T_0 \to T_+ \text{ and emissive T}_0 \to T_- \text{ transitions to
the overall spectrum are plotted separately. The total spectrum, which is the
sum of the two contributions, is shown at the bottom of Figure 3.6. For 3P940,
D/E = 6.5 and the absorptive and emissive patterns are strongly shifted
relative to one another. Their sum is the characteristic A/E/E/A/A/E pattern.
In 3P800, D/E = 2.9, and the absorptive and emissive transitions cancel one
another giving no overall spectrum.
An important consequence of the cancellation of the emissive and absorptive contributions to the RP recombination trEPR spectrum is that it cannot be used as a marker for recombination of the primary RP in randomly oriented samples. However, because the cancellation of the polarization arises because of the overlap of signal contributions from different orientations it will not be complete in oriented samples. For example, the contributions to the spectrum from molecules with their molecular $y$- and $z$-axes parallel to the field cancel each other in a randomly oriented sample. If the molecules can be oriented such that one of these orientations has a higher probability then the cancellation will not be complete and the spectrum should become observable.

3.4.5 trEPR detection of $^{3}P_{840}$ in C. thermophilum membranes

Based on our finding that the absorptive and emissive contributions of a charge recombination triplet will only cancel when the value of $D/E \approx 3$, the $^{3}P_{840}$ spectrum in CabRCs (whose special pair is Zn-BChl $a'/Zn$-BChl $a$) should be detectable by trEPR. In chlorosome-depleted membranes of C. thermophilum, two broad low-intensity triplet features with different $D$ values and with an E/A polarization consistent with ISC formation are observed along with a much narrower E/A RP-like feature at $\sim 345$ mT (Figure 3.7A, red trace). The broad E/A triplet features most likely result
from chlorophyll or carotenoid pigments associated with residual chlorosomes, the carotenoid binding protein and/or the RC bound antenna. If the CabRC containing membranes are frozen in the presence of sodium dithionite at pH 10.0, the narrow RP signal is diminished and replaced with an intense, broad signal with the characteristic polarization pattern (A/E/E/A/A/E) of a charge recombination triplet similar to that of GSbRC (Figure 3.7A, black trace). By taking the derivative of the direct-detection A/E spectrum seen in Figure 3.7A, the values of the zero-field splitting parameters D and E can be obtained (Figure 3.7B, red dotted line). Table 3.3 lists the zero-field splitting parameters for \(^3P_{840}\) in CabRCs as compared to those values calculated for \(^3P_{840}\) in GSbRCs above as well as reported in the literature\(^{10}\). The values of D = 208 x 10\(^{-4}\) cm\(^{-1}\) and E = 35 x 10\(^{-4}\) cm\(^{-1}\) (D/E \(\approx\) 6) are consistent with the identity and delocalization of the \(^3P_{840}\) state over two BChl \(a'\) pigments similar to the BChl \(a'\) dimer in GSbRCs. These results confirm that charge recombination occurs via the triplet state in all photosynthetic RCs.

3.4.6 Oxidation-reduction response of the CabRC RP

The narrow E/A RP signal observed in the wide-field trEPR spectrum shown in Figure 3.7A (red trace) was examined at a higher magnetic field resolution in Figure 3.8A (black spectrum). At 90 K, the RP signal has a peak-
to-peak line-width of 0.8 mT and displays a net absorptive polarization that closely resembles that reported for the HbRC and GSbRC\textsuperscript{10}. Furthermore, the RP can be abolished by chemically oxidizing $P_{\text{840}}$ to $P_{\text{840}^+}$ using 15 mM potassium ferricyanide prior to the trEPR measurement (\textbf{Figure 3.8A}, red spectrum).

When the secondary electron transfer cofactor $F_X$ is reduced or damaged, charge recombination occurs from $A_0^-$ to $P_{\text{840}^+}$ resulting in the observed formation of $^{3}P_{\text{840}}$ and the loss of RP amplitude (\textbf{Figure 3.7A}, black trace). Therefore, if the solution potential is controlled, the redox potential of $F_X$ can be titrated based on the loss of the RP intensity. Using a cocktail of electron mediators, a concentrated solution of dithionite at pH 10.0, and concentrated solution of ferricyanide, CabRCs were poised at various solution potentials from $-283$ mV to $-592$ mV v. SHE. As the solution potential is lowered to $-592$ mV, the amplitude of the RP signal is reduced to approximately 25\% of the value at $-283$ mV (\textbf{Figure 3.8B}). When the amplitude of the RP at the absorptive peak (345.7 mT) is plotted as a function of solution potential, the signal titrates as a one-electron process with a midpoint potential best fit to $-581$ mV ± 7 mV using the Nernst equation (\textbf{Figure 3.8C}).

\textit{3.4.7 Room temperature charge recombination in CabRCs}
To further investigate the electron cofactors on the acceptor side of the CabRC, the kinetics of charge recombination of secondary electron acceptors and $P_{840}^+$ were measured optically following a single-turnover flash at room temperature. When prepared anoxically, CabRCs show biphasic charge recombination with $P_{840}^+$ with lifetimes of 21 ms and 156 ms in roughly equal proportion (Figure 3.9A). These lifetimes are similar to those reported for $P^*F_X^-$ and $P^*F_B^-$ charge recombination events in HbRCs and GsBRCs. Two potential hypotheses may explain why the observed kinetics are not monophasic. 1) Roughly half of the CabRCs lost the $F_A$ and $F_B$ harboring PscB protein during purification. In this case, charge recombination with $P_{840}^+$ proceeds from $F_X^-$ ($\tau \sim 20$ ms) in those RCs without PscB bound and from $F_B^-$ ($\tau \sim 150$ ms) in those that have PscB bound. 2) All of the CabRCs contain PscB, however forward ET from $F_X^-$ to $F_A$ and $F_B$ is not 100% efficient. In this case charge recombination occurs from $F_X^-$ in one fraction of RCs and from $F_B^-$ in the other. Although no direct evidence for the latter hypothesis exists, a similar observation has been noted for HbRCs. Incremental addition of PshBI to a solution of HbRCs with no ferredoxin bound, gradually shows the replacement of the 15 ms $P_{900}^*F_X^-$ phase with a longer 150 ms phase. At a molar ratio of 1:1 (PshBI:HbRC) the kinetics are biphasic with the 15 ms and 150 ms lifetimes in roughly equal proportion.
Interestingly, the addition of excess PshBI does not change the 50:50 proportion of 15 ms to 150 ms charge recombination events, suggesting saturation of the PshBI binding site on the HbRC has occurred. Unfortunately, no expression system exists for heterologously expressing PscB from *C. thermophilum* to carry out a similar experiment. However, the solution conditions can be changed in attempt to remove any of the remaining PscB to determine the likelihood that ~50% was lost during purification.

First, the one-and-the-same sample used in Figure 3.9A was incubated in 500 mM NaCl and 100 mM sodium carbonate at pH 11.5 (to open liposomes) for 30 minutes followed by removal of the soluble fraction by ultracentrifugation. Although similar conditions were sufficient to remove PscB from GSbRCs, P*940* in CabRCs display identical charge recombination kinetics before and after NaCl treatment (Figure 3.9B), suggesting that PscB remained bound. The sample was then incubated with 1 M NaSCN in attempt to remove/denature PscB. After 30 minutes of exposure, the charge recombination kinetics were still biphasic, however the ratio of fast:slow phases shifted slightly in favor of the fast phase (67:33) (Figure 3.9C). After one hour of NaSCN treatment, the total amount of charge-separation was reduced from 4,000 μOD*840 nm* to <1,500 μOD*840 nm*, suggesting PscB as well as Fx may have been lost or damaged (Figure 3.9D). Despite the reduction in
overall charge-separation, some slow charge recombination from PscB is still present. These results strongly suggest that PscB is bound tightly to the CabRC and has not been lost during membrane isolation under only weak ionic conditions (10 mM potassium phosphate buffer with 150 mM NaCl).

3.4.8 Low-temperature charge recombination in CabRCs

Using field-modulated CW EPR in time-sweep mode the lifetime of the steady-state light-induced P$_{840}^+$ cation can also be measured at low-temperature. When the magnetic field is fixed at the low-field peak (344.9 mT) or the high-field trough (345.7 mT) of the light-induced P$_{840}^+$ radical, identical decay kinetics are observed consistent with their representation of the same process (Figure 3.9A). Decay of the high-field peak is best fit as monophasic with a lifetime of 82 ms (Figure 3.9B). This suggests charge recombination proceeds from a single species at low temperatures, unlike the biphasic decay observed at room temperature. This behavior is reminiscent of GSbRCs where forward electron transfer from F$_X$ to F$_A$ and F$_B$ is inefficient at temperatures below 200 K and leads to charge recombination between P$_{840}^+$ and F$_X^-$ with a lifetime of $\sim$30-50 ms$^{10,52}$.

3.5 Discussion

Here we report evidence for the charge recombination triplets of the primary donors from heliobacteria, GSB, and chloracidobacteria. HbRCs
represent a unique case where the charge recombination triplet cannot be observed using trEPR due to the unique, maximally rhombic value of the ZFS parameters, D and E, of its constituent pigment BChl $g'$. In the case where $D = 3E$ complete cancellation of the absorptive and emissive contributions of the trEPR signal occurs in randomly oriented samples. Therefore, optical methodologies such as ODMR must be used to characterize $^{3}\text{P}_{800}$ in HbRCs. In contrast to $^{3}\text{P}_{800}$, which appears to be localized on one BChl $g'$ pigment, $^{3}\text{P}_{840}$ in CabRCs and $^{3}\text{P}_{840}$ in GSB are characterized by similar D and E values consistent with delocalization over both BChl $a'$ pigments in the primary donor (Mg-BChl $a'$ in GSB and Zn-BChl $a'$ in CabRC).

Under non-reducing conditions, CabRCs display a $P_{840}^{*}\text{FX}^-$ RP signal similar to HbRCs and GSbRCs. The net absorptive polarization suggests the lifetime of the $P_{840}^{*}\text{A}_0^-$ state is also similar among homodimeric RCs. This RP is sensitive to the redox state of the RC and can be abolished either by chemically oxidizing $P_{840}$ to $P_{840}^{*}$ with potassium ferricyanide, or chemically reducing FX with sodium dithionite. Titration of the RP suggests the midpoint potential of FX is $-581 \pm 7$ mV v. SHE. This value is $\sim80$ mV more negative than the potential of FX in HbRCs ($-504$ mV)$^{44}$. However, the titration of FX in HbRCs was performed in the absence of bound ferredoxin whereas our optical data suggests at least 50% of the CabRCs contain PscB. Therefore, the
more reducing midpoint potential observed for F_X in CabRCs may reflect an electrostatic effect from the presence of the reduced F_A and F_B clusters within PscB. A similar trend was observed in PSI preparations with and without the F_A and F_B harboring PsaC subunit. In the presence of PsaC, the midpoint potential of F_X was ~50 mV more reducing than when PsaC was absent\textsuperscript{53}.

At room temperature, the lifetime of P\textsubscript{840}+ is composed of fast (21 ms) and slow (156 ms) phases that are similar to the reported P\textsuperscript{+}F_X\textsuperscript{-} and P\textsuperscript{+}F_B\textsuperscript{-} charge recombination events observed in HbRCs and GSbRCs\textsuperscript{2,6}. If the slow (~150 ms) phase is used as a proxy for the presence of PscB, this subunit is only removed following extended exposure to NaSCN at high concentrations (1 M). This suggests PscB is bound more tightly to the CabRC than either of the analogous proteins to the HbRC or GSbRC. This may be important for the observed oxygen stability of the CabRC in in vitro experiments as well as in vivo laboratory culturing of \textit{C. thermophilum}\textsuperscript{42,54}.

At 90 K, the decay of P\textsubscript{840}+ is monophasic with a lifetime of 82 ms. Due to the P\textsubscript{840}+F_X\textsuperscript{-} RP observed by trEPR at this temperature, this likely represents P\textsubscript{840}+F_X\textsuperscript{-} charge recombination. In fact, a similar trend is observed in GSbRCs; below 200 K, ET to F_A and F_B is unfavorable and charge recombination occurs from F_X\textsuperscript{-} at a rate that is 2-3 times slower (\(\tau = 30\)–50 ms) than at room temperature (\(\tau = 17\) ms)\textsuperscript{10,52}. In contrast, the charge
recombination lifetime of P_{880}^* in HbRCs accelerates upon cooling, from 15 ms at 298 K to 2-4 ms below 150 K^{57}. The origin of this accelerated phase however still remains unclear.

Overall, these results confirm that the electron transfer pathways in homodimeric RCs from aerobic and anaerobic phototrophs are highly similar. Despite these similarities, the binding strength of PscB represents an important distinction that raises two questions. 1) Can PscB be removed from the CabRC without damaging F_{X}? 2) Does the presence of PscB modulate the midpoint potential of F_{X}? To address these questions, a systematic study of solution conditions, including chaotropic agents and ionic strengths, should be carried out in attempt to remove PscB from the CabRC. The slow, \sim150 ms charge recombination lifetime, as well as the so far unidentified EPR resonances of F_{A} and F_{B}, can be used as complimentary techniques to track PscB. The quality of the resulting F_{X}-CabRC core could be assayed directly by the presence or absence of the charge recombination triplet \textsuperscript{3}P_{840} using trEPR. For example, if F_{X} is damaged during the removal of PscB, charge recombination will occur between P_{840}^+A_{0}^- resulting in the A/E/E/A/A/E polarization pattern diagnostic of \textsuperscript{3}P_{840}. After PscB is quantitatively removed, the titration of F_{X} can be repeated to determine whether or not the midpoint potential reported here reflects the influence of
the reduced $F_A$ and $F_B$ clusters. Furthermore, if PscB proves amenable to chemical reconstitution with iron and sulfide, the $F_X$-CabRC cores can also be used to examine the effect of binding on the midpoint potentials and oxygen sensitivities of $F_A$ and $F_B$. 
3.6 References


3.7 Figures

Figure 3.1. Jablonski diagram showing the low-temperature electron transfer pathways under oxidizing conditions (left) and reducing conditions (right). The energies and lifetimes of the charge-separated states have been taken from Ferlez et al\textsuperscript{44}. The energy of $^3P_{800}$ has been estimated from the phosphorescence wavelength of BChl $g^{49}$ and the lifetime of formation and decay of $^3P_{800}$ is the literature value at $5\, K^{48}$. 
Figure 3.2. trEPR spectra of GSbRCs (left) and HbRCs (right) under oxidizing and reducing conditions. a: GSbRCs under oxidizing conditions; b: GSbRCs frozen under reducing conditions; c: Difference spectrum b-a; d: HbRCs under oxidizing conditions; e: HbRCs frozen under reducing conditions; f: Difference spectrum e-d. The red spectra are simulations calculated using the parameters in Table 3.1.
Figure 3.3. Fluorescence detected magnetic resonance spectra of HbRCs frozen under oxidizing conditions (green) and under reducing conditions (black).
Figure 3.4. Reconstruction of the FDMR spectrum of HbRCs under strongly reducing conditions (black spectrum) as the sum of three Gaussian components. The red, blue and cyan spectra are the three components and the green spectrum is their sum. The red and blue components are present in oxidized HbRCs but the cyan component due to $^3P_{800}$ only appears when $F_X$ is reduced.
Figure 3.5: Dependence of the spin polarization pattern of a triplet state on the value of the zero-field splitting parameter E, for ISC (left) and RP recombination (right). For all spectra $D = 211 \times 10^{-4} \text{ cm}^{-1}$. For the ISC spectra the triplet sublevels are populated according to their $x, y$ and $z$ character with the population ratios: $p_x : p_y : p_z = 0.5 : 0.5 : 0$. For the RP recombination triplets the high-field states are populated with the ratio $p_{-1} : p_0 : p_1 = 0 : 1 : 0$. 
Figure 3.6: Calculation of the contributions to the polarization pattern generated by radical pair recombination to the triplet state of P$_{840}$ in GsbRCs (left) and P$_{800}$ in HbRCs (right). The spectra have been calculated using the values of D and E given in Tables 3.1 and 3.2.
Figure 3.7: 90 K transient EPR spectra of (A) as isolated (red) and sodium dithionite reduced (pH 10.0) (black) chlorosome depleted *C. thermophilum* membranes. (B) Extraction of zero-field splitting parameters D and E from the charge recombination $^3P_{840}$ observed in chemically reduced chlorosome-
depleted membranes (black solid line) from (A) by measuring the width of the features of its first-derivative (dashed red line). D = 208 \times 10^{-4} \text{ cm}^{-1} \text{ and E} = 35 \times 10^{-4} \text{ cm}^{-1} \text{ (See table 3.3).}
Figure 3.8: Redox response of the P$_{840}^+$F$_X^-$ RP signal measured by transient EPR at 90 K. (A) As isolated (black) and oxidized with 15 mM potassium ferricyanide (red) chlorosome depleted *C. thermophilum* membranes in the presence of 30 mM sodium ascorbate. (B) Representative P$_{840}^+$F$_X^-$ RP spectra at solution potentials of $-283$ mV (solid black line) and $-592$ mV (dashed
black line). (C) Plot of the RP signal intensity measured at 345.7 mT as a function of solution potential. The data is best fit as a one-electron Nernst process with a midpoint potential of $-581 \pm 7$ mV (black solid line). Potentials are displayed versus SHE.
Figure 3.9: Field-modulated time resolved EPR measurement of the lifetime of the P₈₄₀⁺ cation radical at 90 K. (A) Transient decay of P₈₄₀⁺ measured at the low-field peak (344.9 mT) and high-field trough (345.7 mT). Data is the average of 8192 flashes. (B) Decay of P₈₄₀⁺ radical measured at 344.9 mT can be fit as a monoexponential decay with a lifetime of 82 ms.
Figure 3.10: Anoxic room temperature charge recombination kinetics of chlorosome-depleted *C. thermophilum* membranes at 840 nm. (A) Anoxic control sample. (B) Membranes after treatment with 100 mM sodium bicarbonate pH 11.5 and 500 mM NaCl to open liposomes and remove PscB respectively. (C) Membranes after a 30 minute exposure to NaSCN and (D)
Membranes following 1 hour exposure to NaSCN. For experimental details see Materials and Methods (section 3.3.4).
### 3.8 Tables

| Component     | |D| |E| Line-width (mT) | Populations |
|---------------|--------------|--------------|----------------|--------------|
| GSbRC antenna | 306          | 37           | 8.8            | $p_x:p_y:p_z = 0.13:0.87:0$ |
| GSbRC $^3P_{4/2}$ | 208          | 32           | 1.3            | $p_{-1}:p_0:p_1 = 0:1:0$ |
| HbRC antenna  | 227          | 77           | 2.0            | $p_x:p_y:p_z = 0.62:0.38:0$ |
| HbRC antenna  | 233          | 82           | 2.0            | $p_x:p_y:p_z = 0.62:0.38:0$ |

**Table 3.1:** Simulation parameters for the trEPR spectra in **Figure 3.2.**
| Component | |D|-|E| Width | |D|+|E| Width | |D|× | |E| | Q_y |
|----------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Antenna  | 448 MHz          | 14 MHz           | 912 MHz          | 21 MHz           | 226.8 MHz        | 77.4 MHz         | 815 nm           |
| Antenna  | 453 MHz          | 14 MHz           | 944 MHz          | 25 MHz           | 233.0 MHz        | 81.9 MHz         | 798 nm           |
| \(^3\)P_{800} | 475 MHz          | 11 MHz           | 980 MHz          | 13 MHz           | 242.7 MHz        | 84.2 MHz         | 792 nm           |

Table 3.2: Zero-field splitting parameters of HbRCs from FDMR data.
<table>
<thead>
<tr>
<th>Zero-Field Splitting Parameters</th>
<th><em>C. thermophilum</em> (this study)</th>
<th><em>C. tepidum</em> (this study)</th>
<th><em>C. tepidum</em> Literature¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90 K (x 10⁻⁴ cm⁻¹)</td>
<td>90 K (x 10⁻⁴ cm⁻¹)</td>
<td>80 K (x 10⁻⁴ cm⁻¹)</td>
</tr>
<tr>
<td><strong>D</strong></td>
<td>208</td>
<td>208</td>
<td>208</td>
</tr>
<tr>
<td><strong>E</strong></td>
<td>35</td>
<td>32</td>
<td>33</td>
</tr>
</tbody>
</table>

**Table 3.3:** Zero-field splitting parameters (ZFS) measured from charge recombination triplets of $^3P_{840}$ in *C. thermophilum* (Figure 3.7) and *C. tepidum* (Figure 3.2). For literature reference, see a, van der Est et al¹⁰.
Chapter 4

The Effect of Bacteriochlorophyll $g$ Oxidation on Energy and Electron Transfer in Reaction Centers from *Heliobacterium modesticaldum*

Published as:
4.1 Abstract

The heliobacteria are a family of strictly anaerobic, gram positive, photoheterotrophs in the Firmicutes. They make use of a homodimeric Type I reaction center (RC) that contains ~18 antenna bacteriochlorophyll (BChl) \( g \) molecules, a special pair of BChl \( g' \) molecules \( (P_{800}) \), two \( 8^1\)-OH-Chl \( a_F \) molecules \( (A_0) \), a \([4Fe-4S]^{2+/1+}\) iron-sulfur cluster \( (F_X) \), and a carotenoid \( (4,4'-\text{diaponeurosporene}) \). It is known that in presence of light and oxygen BChl \( g \) is converted to a species with an absorption spectrum identical to that of Chl \( a \) \( (\text{Chl} \ a_{ox}) \). Here, we show that main product of the conversion is \( 8^1\)-OH-Chl \( a_F \). Smaller amounts of two other oxidized Chl \( a_F \) species are also produced.

In the presence of light and oxygen, the kinetics of the conversion are monophasic and temperature dependent, with an activation energy of \( 66\pm2 \text{ kJ mol}^{-1} \). In the presence of oxygen in the dark, the conversion occurs in two temperature-dependent kinetic phases: a slow phase followed by a fast phase with an activation energy of \( 53\pm1 \text{ kJ mol}^{-1} \). The loss of BChl \( g' \) occurs at the same rate as the loss of BChl \( g \), hence the special pair converts at the same rate as the antenna BChls. However, the loss of \( P_{800} \) photooxidiation and flavodoxin reduction are not linear with the loss of BChl \( g \). In RCs incubated and frozen under anoxic conditions the charge recombination between \( P_{800}^+ \) and \( F_X^- \) at 80 K is monophasic with a lifetime of 4.2 ms but after exposure to
oxygen an additional phase with a lifetime 0.3 ms is observed. Transient EPR (trEPR) data show that the line-width of $P_{800}^+$ increases as BChl $g$ is converted to Chl $a_{ox}$ and the rate of electron transfer from $A_0$ to $F_X$, as estimated from the net polarization generated by singlet-triplet mixing during the lifetime of $P_{800}^*A_0^-$, is unchanged. The trEPR data also show that conversion of BChl $g$ results in increased formation of triplet states of both BChl $g$ and Chl $a_{ox}$. The nonlinear loss of $P_{800}$ photooxidation and flavodoxin reduction, the biphasic charge recombination kinetics and increased EPR line-width of $P_{800}^+$ are all consistent with a model in which the BChl $g'/BChl$ $g'$ and the BChl $g'/Chl$ $a'_{ox}$ special pairs are functional, but the Chl $a'_{ox}/Chl$ $a'_{ox}$ special pair is not.

4.2 Introduction

Heliobacteria are anoxygenic phototrophic bacteria that contain a homodimeric Type I RC\textsuperscript{1}. Unlike green sulfur bacteria or chloracidobacteria, they do not contain chlorosomes or antenna complexes and rely solely on pigments bound to the RC for light-harvesting\textsuperscript{2}. Compared to Photosystem I (PSI) of cyanobacteria and plants, the heliobacterial reaction center (HbRC) contains fewer pigments and protein subunits and, as a result, it is an ideal model for the study of a simple, prototypical homodimeric RC\textsuperscript{2}. In the HbRC $\sim 18$ BChl $g$ molecules function as an antenna along with the carotenoid $4,4'$-
diaponeurosporene\textsuperscript{4-7}. The primary electron donor, P\textsubscript{800}, is a special pair of BChl \textit{g} molecules (the C-13\textsuperscript{2} epimer of BChl \textit{g}), while the primary acceptor, A\textsubscript{0}, is 8\textsuperscript{1}-OH-chlorophyll \textit{a}\textsubscript{F} (where the subscript \textit{F} stands for farnesyl)\textsuperscript{5,8,9}. Unlike PSI, the HbRC does not contain a tightly bound dicluster ferredoxin as a subunit\textsuperscript{10}. Instead, the F\textsubscript{X} cluster functions as the terminal acceptor, donating electrons to a variety of soluble redox partners, including the loosely-bound dicluster ferredoxins PshBI and PshBII\textsuperscript{11}. The HbRC also contains menaquinone, but its function remains unclear\textsuperscript{6,12}. Time-resolved optical and EPR data show that electron transfer between A\textsubscript{0} and the iron-sulfur (FeS) cluster F\textsubscript{X} occurs with a lifetime of \textasciitilde700 ps and there is no evidence from kinetic lifetimes for an intermediate between these two acceptors\textsuperscript{13-19}. However, EPR signals assigned to a photo-reduced quinone radical have been reported in samples that have been treated with dithionite to reduce F\textsubscript{X}\textsuperscript{20-22}.

Heliobacteria grow only under strictly anoxic conditions, and when exposed to air, BChl \textit{g} is oxidized to a species with an absorbance spectrum identical to that of Chl \textit{a} (Chl \textit{a}\textsubscript{ox})\textsuperscript{23,24} (Figure 4.1). In cells and isolated membranes, the two Soret transitions, the Q\textsubscript{x} and Q\textsubscript{y} bands of BChl \textit{g} occur at 368, 409, 575, and 788 nm, respectively\textsuperscript{25,26}. On exposure to air and light the
Q_y transition shifts to 670 nm\textsuperscript{23}. This shift can be used to conveniently monitor the conversion by optical absorption spectroscopy\textsuperscript{24}.

The chemical structure of the oxidation product (or products) and the effect of this conversion on the function of HbRCs have not been adequately investigated. Recently, an NMR study demonstrated that partial conversion of BChl g does not impair the appearance of dynamic nuclear polarization (photo-CIDNP) resonances, indicating that light-induced charge-separation takes place\textsuperscript{27}. This system therefore presents a unique opportunity to investigate the influence of chlorophyll energetics on the kinetics and quantum yield of energy and electron transfer, since the alteration of the chlorophyll does not require any mutagenesis or cofactor exchange. Moreover, the conversion is not expected to significantly perturb the arrangement of the chlorophylls and their binding sites within the protein matrix while still causing a large change in the energetics as evidenced by the shift of nearly 100 nm in the Q_y absorbance transition. However, before the effect of the conversion on the function of the RC can be studied in detail, the conversion itself must be better understood. Here, we report a systematic study of the conversion of BChl g and a first investigation of its effect on energy and electron transfer in homodimeric Type I RCs isolated from \textit{H. modesticaldum}. 
4.3 Materials and Methods

4.3.1 Growth and isolation of HbRC cores

Cultures of *Heliobacterium modesticaldum* were generously provided by Prof. Mike Madigan (Southern Illinois University, Carbondale, IL). Liquid cultures of *H. modesticaldum* were grown under anoxic conditions in PYE media\textsuperscript{28}. Resazurin, a reporter dye, was added to a final concentration of 0.001%. All manipulations were performed under anoxic conditions. Cells grown to late-exponential phase were harvested at 10,000 x g for 30 min and resuspended in 50 mM MOPS buffer (pH 7.0). Whole cells were lysed by sonication, and unbroken whole cell were removed by centrifugation at 10,000 x g. The dark brown supernatant, which contains the membranes, was pelleted by centrifugation at 200,000 x g and solubilized with 1% *n*-dodecyl-β-D-maltopyranoside (DDM) for 1 hour. Unsolubilized membranes were removed by centrifugation at 200,000 x g for 30 min. The supernatant was passed over a diethylaminoethyl (DEAE) cellulose ion-exchange column equilibrated in 50 mM MOPS containing 0.02% DDM (pH 7.0). This removes the PshBI and PshBII polypeptides that contain the F\textsubscript{A} and F\textsubscript{B} clusters. The flow through contained the HbRC cores in which F\textsubscript{X} is the terminal electron acceptor\textsuperscript{17}. The HbRC cores were concentrated by ultrafiltration and stored in liquid nitrogen until use.
4.3.2 UV-visible absorption spectroscopy

UV-visible absorption spectroscopy was carried out using a Varian Cary 50 spectrophotometer. All spectra were corrected for background and offset effects. The spectra of HbRC cores were measured in 3 mL quartz cuvettes with a 10 mm path length at an optical transmittance of 0.8 OD at 788 nm. A 25 W tungsten lamp coupled with a fiber optic cable (diameter = 1 cm) was used to drive the photo-oxidation of BChl g at temperatures ranging from 297 K to 319 K and spectra were recorded every 10 minutes.

4.3.3 HPLC-mass spectrometry of RC pigments

High-performance liquid chromatography (HPLC) with detection by mass spectrometry (LC-MS) was conducted on an Agilent Technologies (Santa Clara, CA) 1200 system coupled to an Agilent Technologies 6410 QQQ mass spectrometer. The system was operated with the associated MassHunter software package, which was also used for data collection and analysis.

Pigments were extracted from HbRC preparations (3.2 mM BChl g) in acetone/0.1% formic acid (pH 2.6) and were separated on an Agilent Technologies Zorbax Extend-C18 RRHT column (4.6 mm × 50 mm, 1.8 µm particle size) equilibrated in 100% solvent A (74:6:1 acetonitrile:methanol:formic acid, pH 2.6). After two minutes at 100%
solvent A, a gradient of 0 to 100% solvent B (5:1 methanol:acetonitrile) was applied from 2 to 4.5 min and maintained at 100% solvent B from 4.5 to 9 min before returning to 0% solvent B from 9 to 10 min. A flow rate of 0.5 mL/min was maintained throughout the chromatographic procedure. The column was allowed to re-equilibrate for 3 min under initial conditions before subsequent sample injections. Prior to quantification of the relative BChl g and BChl g′ content, the HPLC traces were normalized to the content of the carotenoid 4,4'-diaponeurosoporene (m/z 402.5), which elutes at approximately 6.4 min under these conditions.

BChl g and oxidized Chl a F derivatives were detected by MS2 selected-ion monitoring at m/z ratios of 811.5, 819.5, 835.5 and 851.5. Detection of analytes was performed by selected ion monitoring (SIM) using electrospray-ionization in positive mode (ESI+) with a nitrogen gas temperature of 350 °C and flow rate of 9.0 L/min, a nebulizer pressure of 40 psi, and a capillary voltage of 4000 V. The optimal fragmentor voltage for BChl g and oxidized Chl a F derivatives was determined to be 90 V while the optimal fragmentor voltage for the 4,4'-diaponeurosoporene was determined to be 150 V.

UV-visible absorbances were collected at 752 (20), 662 (20), 410 (20), 464 (20), and 280 (8) nm (bandwidth denoted in parentheses). UV-visible
spectra were collected from 260 to 900 nm in 2 nm steps every 0.5 s using the associated diode array detector.

4.3.4 Flavodoxin reduction assay

Dilute HbRCs at 70 µM [BChl g] were illuminated with a xenon lamp at an intensity of 224 µmol photons m\(^{-2} s\(^{-1}\) to convert BChl g to Chl \(a_{ox}\). At each time point a sample was removed, sealed, and purged with argon gas at 6 psi for 15 minutes to remove \(O_2\) from the solution. Flavodoxin reduction assays were then carried out as described in detail in Romberger and Golbeck\(^{11}\). Briefly, the change in the absorbance of flavodoxin was monitored at 467 nm while the sample was illuminated using white light. The rate of flavodoxin reduction was determined by a linear fit to the absorbance change over the first 1-2 s of illumination. Each flavodoxin measurement is the average of three data collections. For the light saturation curves, samples were illuminated with a continuous-wave (CW) Verdi V5 laser at 532 nm passed through a 3X optical beam expander. The change in absorbance at 467 nm was recorded after two minutes of illumination. Saturation curves were fit with the Michaelis-Menton equation using a nonlinear least squares fitting algorithm in Igor Pro. The percent of BChl \(g\) converted was determined by comparison of the \(Q_y\) band of BChl \(g\) as a function of light and \(O_2\) exposure.
4.3.5 $P_{800}$ photobleaching and time-resolved optical spectroscopy

$P_{800}$ photobleaching was measured on a JTS-10 pump probe spectrometer (BioLogic) using a continuous orange ring LED actinic light source ($\lambda_{max} = 640$ nm) and a pulsed probe beam provided by an 810 nm LED. To monitor photobleaching at 800 nm an interference filter was placed between the probe beam and the sample. HbRC samples were prepared by dilution with 50 mM MOPS pH 7.0 with 0.02% DDM and 10 mM sodium ascorbate with a final [BChl $g$] of 400 $\mu$M.

To determine the differential absorption spectrum of the RC following conversion of BChl $g$ to Chl $a_{ox}$ the amplitude of the light-induced absorbance change of an anoxic and 89% converted sample was measured 10 $\mu$sec after a saturating laser flash provided by a frequency doubled Nd-YAG laser (SpectraPhysics). Using light from a tungsten lamp passed through a monochromator as well as an interference filter, absorbance changes were measured every 10 to 20 nm from 509 to 910 nm. Each point is the average delta absorbance from 64 collections. Sample composition was 10 $\mu$M BChl $g$ and 10 mM sodium ascorbate in 50 mM MOPS pH 7.0 with 0.02% DDM.

4.3.6 Transient EPR experiments

Time/field trEPR datasets were collected using a modified Bruker ER 200D-SRC spectrometer with either an ER 041 X-MR X-band or an ER 051 QR
Q-band microwave bridge. For the X-band datasets, a Bruker Flexline ER 4118 X-MD-5W1 dielectric resonator was used. A Bruker ER 5106 QT-W cylindrical resonator was used for the Q-band experiments. Excitation was achieved using a Continuum Surelite Nd-YAG laser operating at 10 Hz and a wavelength of 532 nm and 4.0 mJ/pulse. The temperature was controlled using an Oxford Instruments CF935 gas flow cryostat. The spin-polarized transient EPR signals of the chlorophyll triplet states and radical pair $P_{800}^+F_X^-$ were collected in direct-detection mode with a home-built broadband amplifier (bandwidth > 500 MHz). For measurements of the lifetime of $P_{800}^+$, the signal was collected using lock-in detection and a Bruker ER032 signal channel. In both cases the signals were digitized using a LeCroy LT322 500 MHz digital oscilloscope and saved on a computer for analysis. For the EPR experiments HbRCs were exposed to air in the dark at ambient temperature for varying amounts of time up to 12 hr at which point dissolved oxygen was removed by purging each sample with argon gas at 6 psi for 15 minutes. Samples were then moved to an anoxic chamber (5% hydrogen, 95% nitrogen) (Coy Labs) and the sample was transferred to EPR tubes and frozen in the dark with liquid nitrogen.
4.4 Results

4.4.1 Absorption changes in the HbRC under illumination with dioxygen as oxidant

Recently, it was reported that the HbRC could be handled for extended periods of time in oxic conditions with dim green light without significant conversion of BChl $g^5$. Thus, it is of interest to better understand the effect of light, oxygen, and temperature on the conversion of BChl $g$ to Chl $a_{ox}$. The absorption spectra of RC cores in 50 mM MOPS buffer (pH 7.0) at 298 K in the presence of O$_2$ and light are shown at 10-minute intervals of illumination in Figure 4.2A. Over the course of two hours, the characteristic peaks of BChl $g$ at 788, 575 and 368 nm decline, while the characteristic peaks of Chl $a_{ox}$ at 670 and 438 nm increase. During this time, the appearance of the solution changes from brown to emerald green. The presence of multiple isosbestic points, especially prominent at 695 nm, 595 nm and 480 nm, indicates that the conversion occurs without the accumulation of an apparent intermediate. The decline of the intensity of the 788 nm peak from BChl $g$ and rise of the intensity of the 670 nm peak from Chl $a_{ox}$ are plotted against time on a logarithmic scale in Figure 4.2B. (For the 788 nm peak the absorbance relative to the initial intensity is plotted. For the 670 nm peak, the absolute absorbance increase is shown.) The loss of BChl $g$ is exponential with time and mirrors the increase in Chl $a_{ox}$. At room temperature (298 K), two hours
are required for 63% of BChl g to be converted to Chl \( a_{\text{ox}} \), which corresponds to a rate constant of \( 1.01 \times 10^{-4} \) s\(^{-1}\). The loss in the amplitude of BChl g at 788 nm, measured at five temperatures is shown in Figure 4.2C and an Arrhenius plot of the rate constants (Figure 4.2D) yields an activation energy of \( 66 \pm 2 \) kJ mol\(^{-1}\) for the light-mediated conversion of BChl g to Chl \( a_{\text{ox}} \) in the presence of O\(_2\).

4.4.2 Absorption changes in the HbRC in darkness with dioxygen as oxidant

The absorption spectra of HbRCs incubated in the dark at 298 K in the presence of O\(_2\) are shown in Figure 4.3A. Similar to the study carried out in light, the characteristic peaks of BChl g at 788, 575 and 368 nm decreased, while the characteristic peaks of Chl \( a_{\text{ox}} \) at 670 and 438 nm increased and the presence of the same isosbestic points indicates that no detectable intermediate accumulates during the conversion. The decline of the intensity of the 788 nm peak from BChl g and the rise of the intensity of the Chl \( a_{\text{ox}} \) at 670 nm are plotted against time on a logarithmic scale in Figure 4.3B. The conversion of BChl g to Chl \( a_{\text{ox}} \) can be seen to occur in two distinct phases. Over the first three hours, the 788 nm peak decreases slowly from 1.07 OD to 0.98 OD, during which time only 8% of BChl g is converted to Chl \( a_{\text{ox}} \). At the three-hour point, a seemingly abrupt transition occurs, and the absorbance
decreases rapidly from 0.98 OD to 0.44 OD over the course of 6 hours, during which time approximately 45% of BChl g is converted to Chl \( a_{ox} \). The absorbance decay during the initial slow phase could not be fitted easily with a simple kinetic model but the fast decay is exponential and the activation energy of this phase was determined. Figure 4.3C shows the temperature dependence of the fast phase of the 788 nm absorbance loss. For each temperature, the point at which the transition from the slow to fast phase was deemed to be complete (Table 4.1) was chosen as the zero point on the time axis for the data in Figure 4.3C. The Arrhenius plot of the rate constants (Figure 4.3D) gives an activation energy of 53 ± 1 kJ mol\(^{-1}\) for the fast phase in the conversion of BChl g to Chl \( a_{ox} \) in the dark.

4.4.3 Effect of vitamin K3 on the \( O_2 \)-mediated conversion of BChl g to Chl \( a_{ox} \) in the dark

Menadione (Vitamin K3) significantly retards the rate of the dark \( O_2 \)-mediated conversion of BChl g to Chl \( a_{ox} \). At an equimolar ratio of menadione to HbRC, the absorbance at the 788 nm peak of BChl g decreases from 0.75 OD to 0.65 OD after 20 hours of exposure to \( O_2 \). This loss of absorbance corresponds to a conversion of only 13% of the bulk BChl g pigment, which is significantly less than the 49% conversion observed in a sample of identical HbRCs exposed to \( O_2 \) in the absence of menadione. The conversion of BChl g to Chl \( a_{ox} \) could be further arrested to the point that no conversion could be
detected over 20 hours by increasing the molar ratio of menadione to RC to 30:1 (data not shown).

4.4.4 HPLC-mass spectrometry of pigments during dark dioxygen exposure

The multiphasic kinetics of the conversion in the dark suggests that exposure to O$_2$ does not just lead to simple isomerization of BChl $g$ to Chl $a_{ox}$ and it is possible that multiple oxidation products are formed as observed previously for the conversion in diethyl ether and other solvents$^{24}$. Hence, we performed an HPLC-MS analysis to determine which species are present in the samples. This analysis also allows of the rate of conversion of the BChl $g'$ pigments, which constitute the P$_{800}$ special pair, to be compared to that of the BChl $g$ cofactors. The total pigment content extracted from HbRCs that had been exposed to O$_2$ for varying times in the dark corroborates the conversion of BChl $g$ to Chl $a_{ox}$ shown above in the UV-visible experiments. Consistent with previously published results, four major peaks are observed in the control sample prior to exposure to O$_2$ (Figure 4.4A, peaks 2, 4, 5, 8), and these can be assigned to: 8$^1$-OH Chl $a_F$, BChl $g$, BChl $g'$ and 4,4'$'$-diaponeurosporene respectively (Figure 4.4B)$^5$. Small amounts of residual O$_2$ in the sample or extraction buffer (0.1% formic acid/acetone) likely lead to the accumulation of some isomerized BChl $g$ (Figure 4.4A, peak 7 - Chl $a_F$), consistent with the acid-catalyzed mechanism proposed by Kobayashi et al$^{24}$. 
The relative amount of this species is independent of the loss of BChl $g$ and therefore is not related to the dark conversion that occurs prior to extraction. After 6 hours of exposure to O$_2$, the content of 8$^1$-OH-Chl $a_F$ (Figure 4.4A, peak 2) increases and three new peaks are observed (Figure 4.4A, peaks 1, 3, 6). Peak 1 grows in as a distinct shoulder on the leading edge of peak 2 and has an absorption spectrum and mass to charge ratio (m/z) that suggests a Chl $a_F$ species with the addition of two hydroxyl groups. The absorption spectra and m/z of peak 3 suggest it is another Chl $a_F$ derivative with the addition of a single oxygen atom. Hence, the conversion product of BChl $g$ after exposure to O$_2$ is 8$^1$-OH-Chl $a_F$ with an additional small amount of what is tentatively identified as 8$^1$-geminal-diol-Chl $a_F$ and its dehydration product, 8$^1$-keto-Chl $a_F$. All three oxidation products of BChl $g$ will be collectively termed Chl $a_{ox}$ in this chapter. Peak 6 also has an absorption spectra and m/z that is similar to peak 1 which suggests it is also a Chl $a$ derivative; however its retention time is unexpectedly longer (ca. 5.5 min) and further study is required to determine its origin.

The total BChl $g$’ content (Figure 4.4A, peak 5) of the sample can be seen to decrease linearly with the loss of BChl $g$ (Figure 4.4C) When the integrated area of this peak is plotted as a function of the total BChl $g$ converted, the data can be fit with a straight line with a slope of $-1.1$ and an
r² value of 0.99. Thus, the special pair BChl $g'$ molecules are nearly equally susceptible to oxidative conversion as the antenna BChl $g$ molecules.

4.4.5 Flavodoxin reduction and transient optical spectroscopy during conversion

The rate of flavodoxin reduction was compared to the extent of photobleaching of the primary donor P₈₀₀ at various points in the conversion of BChl $g$ to Chl $a_{ox}$. The rates of flavodoxin reduction were determined from the time dependence of associated absorbance change at 467 nm, while the photobleaching of the primary donor was monitored at 800 nm. The flavodoxin reduction reflects the rate at which the RCs are able to withdraw electrons from the soluble donor 1-methoxy-5-methylphenazinium methyl sulfate (PMS) and transfer them to the soluble acceptor flavodoxin, whereas the photobleaching of P₈₀₀ is a measure of the yield of charge-separation between P₈₀₀ and Fₓ. As shown in Figure 4.5A, both of these measures of the activity of the RCs show the same dependence on the degree of BChl $g$ conversion. To distinguish between inefficient energy transfer and loss of electron transfer, the dependence of the flavodoxin reduction on the light intensity was studied. As the light intensity is increased the amount of flavodoxin reduced ($\Delta$Abs₄₆₇nm) follows a saturation curve. The efficiency of the energy transfer is reflected in amount of light needed to reach saturation and the maximum amount of flavodoxin reduced reflects the electron
transfer efficiency. Impairment of the energy transfer results in more light being required to reach saturation and lower electron transfer efficiency results a lower amount of reduced flavodoxin at saturation. Light saturation curves were measured for the 0% and 70% BChl g converted samples (Figure 4.5B). The half-saturation light intensity for the 70% converted sample (39 mW) is ca. four times greater than that for the 0% converted sample (11 mW), indicating impaired efficiency of energy transfer among the antenna pigments (see section 3.10). Nevertheless, the amount of reduced flavodoxin saturates at a lower value for the 70% converted sample (0.099 ΔAbs467nm) than the 0% converted sample (0.135 ΔAbs467nm), showing that at saturation, activity is lost due to loss of charge-separation.

Figure 4.5A shows that the dependence of the flavodoxin reduction and amplitude of P800 bleaching on the degree of BChl g (or BChl g′) conversion is nonlinear. For example, at 30% BChl g conversion, there is no appreciable loss of flavodoxin reduction and P800* formation. This behavior is not consistent with a model in which conversion of one of the special pair BChl g′ molecules is sufficient to inactivate the HbRC. The observed dependence on the degree of conversion can be explained in the following manner. The distribution of possible pigment combinations in the primary donor, (BChl g′/BChl g′), (Chl a′ox/BChl g′) or (Chl a′ox/Chl a′ox) can be
determined from the degree of conversion of BChl $g'$. Since the rates of conversion of BChl $g$ and BChl $g'$ are equal (Figure 4.4C), the overall degree of conversion of BChl $g$ can be used. The population of each of the three possible forms of the primary donor is given by the product of the fractions of each of the pigments in the RC. As shown in Figure 4.5C, the BChl $g'/BChl
g'$ containing special pair is predicted to decreases with conversion (brown line), while the (Chl $a'_ox$/Chl $a'_ox$) containing special pair increases with conversion (green line). In both cases the conversion follows a quadratic relationship. In contrast, the concentration of the heterodimeric special pair (BChl $g'/Chl
g'$) increases to a maximum at 50% conversion and then decreases thereafter (black line). The sum of the concentrations of the (BChl $g'/BChl
g'$) and (BChl $g'/Chl
ga'_ox$) forms gives the red curve in Figure 4.5C. Since the activity of the RCs eventually decreases to zero as the conversion nears completion we can safely assume that the (Chl $a'_ox$/Chl $a'_ox$) form of the special pair is inactive. Hence, the activity should follow the brown curve from Figure 4.5C if conversion of one of the two chlorophylls is sufficient render the RC inactive and the red curve if both chlorophylls must be converted. As can be seen in Figure 4.5A, the red curve fits well the experimental activity data observed for both flavodoxin reduction and P$_{800}$ photobleaching. This suggests that the loss of activity stems exclusively from
the fraction of RCs in which both primary donor pigments have been converted (i.e. Chl \( a'_{\text{ox}} \)/Chl \( a'_{\text{ox}} \)) (Figure 4.5C, green line). Consistent with this assessment, when BChl \( g \) is 89% converted to Chl \( a_{\text{ox}} \), no significant photobleaching is detected implying that no long-lived charge separated trap is formed at wavelengths characteristic of Chl \( a \) (670 nm) (Figure 4.5D).

4.4.6 Lifetime of the charge-separation at 80 K

Figure 4.6 shows time-resolved EPR time traces of \( P_{800}^+ \) collected at 80 K using field modulation detection. In the sample that has not been exposed to \( O_2 \) (Figure 4.6A) the signal decays mono-exponentially with a lifetime of 4.2 ± 0.5 ms. Exposure to \( O_2 \) and the accompanying conversion of BChl \( g \) to Chl \( a_{\text{ox}} \) leads to a decrease in the overall intensity of the signal and the appearance of an additional fast component with a lifetime of 0.3 ± 0.1 ms. The appearance of a fast charge recombination kinetic indicates that at least one of the electron transfer cofactors has been altered and it is likely that it occurs in HbRCs in which one of the BChl \( g' \) molecules of \( P_{800} \) has been converted to Chl \( a'_{\text{ox}} \). The relative amplitudes of these two components as a function of the exposure time are shown in Figure 4.6B. As can be seen, the amplitude of the slow component (red circles) decays with increasing exposure, while the fast component (blue circles) initially increases and then decreases. The sum of the two amplitudes (black circles) decreases.
Qualitatively, the 4.2 ms component appears to follow the predicted content of the BChl $g'/BChl$ $g'$ special pair, and the 0.3 ms component follows the predicted content of the BChl $g'/Chl$ $a'_{ox}$ special pair. However, the exposure to $O_2$ and illumination at 80 K also leads to accumulation of a stable $g \approx 2$ signal probably due to $P_{800}^+$. The amplitude of this signal is plotted in Figure 4.6C. Because the kinetic phases represent the response to single flashes, while the stable $P_{800}^+$ signal accumulates with repeated flashes, a quantitative analysis of their amplitudes in terms of the percent conversion of BChl $g$ and possible forms of the special pairs in the sample is not feasible.

### 4.4.7 Spin-polarized EPR spectrum of $P_{800}^+F_X^-$ at 80 K

The presence of two charge recombination phases indicates that the $O_2$-exposed samples contain a population of functionally active RCs in which one or more of the cofactors have been altered. This raises the question of whether the rate of forward electron transfer to $F_X$ is altered and whether the spectroscopic properties $P_{800}^+$ are changed. Both of these questions can be addressed by trEPR experiments. Figure 4.7 shows spin-polarized trEPR spectra of $P_{800}^+F_X^-$ taken at 80 K at X-band (A) and Q-band (B). The spectra arise from transitions associated with $P_{800}^+$ and show an E/A polarization pattern. The absorptive parts of the spectra are stronger than the emissive parts indicating that the spin system has net absorptive polarization as a
result of singlet-triplet (S-T) mixing in the precursor state \( P_{800}^* A_0^- \). An analytical treatment of sequential radical pairs shows that the polarization can be broken down into three main components\(^{29}\). A purely multiplet contribution (equal amounts of emission and absorption) from singlet electron transfer, a net contribution due to S-T mixing during the lifetime of the precursor, and a multiplet contribution to the polarization of only the donor cation due to the influence of inhomogeneous linebroadening (e.g. from unresolved hyperfine couplings) on the S-T mixing. The intensity of the net contribution is given by:

\[
I_{\text{net}} = \frac{q b_0}{k^2}
\]  

(1)

where \( q \) is the difference of the precession frequencies of the two spins in the primary radical pair, \( b = 2d + J \) is the spin-spin coupling and \( k \) is the rate of electron transfer from \( A_0^- \) to \( F_X \). The hyperfine contribution is:

\[
I_h \propto \frac{b_0 D}{k^2}
\]  

(2)

where \( D \) is the inhomogeneous line-width of the donor.

Using this model, the polarization patterns of anoxic samples from heliobacteria can be reproduced well by taking into account the mixing that occurs during the \( \sim 600 \) ps lifetime of the precursor state \( P_{800}^* A_0^- \). The
simulated spectra of the anoxic sample in Figure 4.7 (red curves at 0 hr exposure) are calculated using the parameters derived from Photosystem I as reported previously, except that the line-width associated with P_{800^*} is 0.87 mT instead of the value of 0.80 mT used in the earlier simulations^{31}. As can be seen, the absorptive net polarization (difference in intensity of the absorptive and emissive peaks) is much larger in the Q-band spectra (Figure 4.7B) than in the X-band spectra (Figure 4.7A) as predicted by Eqn. 1. Upon exposure to oxygen the intensity of the signal drops but the overall line-width increases noticeably and the amount of net polarization relative the multiplet polarization is significantly less. Because the multiplet contribution associated with hyperfine coupling induced S-T mixing depends on the line-width of the donor (Eqn. 2), while the net polarization does not, an increase in the line-width leads to an increase in the amplitude of the multiplet polarization relative to the net polarization. Based on the biphasic charge recombination kinetics, the P_{800} photobleaching, and the flavodoxin reduction studies, we expect that the spectra of the samples that have been exposed to O_2 arise from a mixture of radical pairs with either (BChl g'/ BChl g') or (BChl g'/Chl a'ox) as the special pair. If the line-width of P_{800^*} in the (BChl g'/Chl a'ox) special pair is taken as 1.2 mT and the relative amounts of the two radical pairs is taken from the ratio of the fast and slow phases of
charge recombination, good agreement with the experimental spectra is obtained as can be seen in the simulations for the sample exposed to oxygen for 8 h shown in Figure 4.7. From this we can conclude that the population of RCs with fast charge recombination kinetics has a special pair with a larger inhomogeneous line-width. Such an increase in line-width is expected in a heterodimeric special pair due to localization of the unpaired spin density on one of the two chlorophylls. However, other factors such as changes in the T2 relaxation time and altered matrix hyperfine couplings could also account for the difference in line-width. Thus, further experimental data such as ENDOR spectra of P800+ are needed to determine the origin of the line-width change.

4.4.8 Triplet state formation at 80 K

Figure 4.8A shows experimental spin-polarized chlorophyll triplet state spectra of the HbRC taken at 80 K and calculated spectra fitted to the experimental ones collected by trEPR. In the absence of O2, an emission/absorption (E/A) pattern ~60 mT wide triplet state spectrum is observed. The narrow E/A ~4 mT wide features are from the radical pair P800+F-X-. The polarization pattern of the triplet spectrum indicates that the triplet state is formed by intersystem crossing and the values of the zero field splitting parameters $D = 215 \times 10^{-4}$ cm$^{-1}$, $E = 72 \times 10^{-4}$ cm$^{-1}$ are consistent with those reported previously$^{32, 33}$ for the triplet state of BChl g. Upon
exposure to $O_2$ for 12 h in the dark, additional features appear in the spectrum. The additional contribution is consistent with the triplet state of a Chl $a$ species with $D = 271 \times 10^{-4}$ cm$^{-1}$, $E = 33 \times 10^{-4}$ cm$^{-1}$ and a polarization pattern that is determined by intersystem crossing. Very weak features in the wings of the spectrum suggest that there may also be a small contribution from carotenoid triplet states as well. The spectra of all of the $O_2$-exposed samples can be simulated as a weighted sum of the BChl $g$ and Chl $a_{ox}$ spectra shown at the bottom of Figure 4.8A. The amplitudes of these contributions relative to the amplitude of the triplet spectrum in the anoxic sample are plotted in Figure 4.8B. As can be seen, the intensities of both contributions increase with increasing exposure time. These results suggest that the gradual conversion of BChl $g$ to Chl $a_{ox}$ has two effects. The appearance of a Chl $a_{ox}$ triplet spectrum implies that at 80 K, the converted molecules are unable to transfer their excitation energy. The increase in the amplitude of the BChl $g$ triplet signal suggests that some of the antenna BChl $g$ molecules that would otherwise transfer their excitation energy to neighboring molecules are unable to do so. However, it is unclear whether this is the result of the conversion of neighboring BChl $g$ molecules or the presence of closed RCs in which $P_{800}$ has been trapped in an oxidized state.
4.5 Discussion

The conversion of BChl $g$ to Chl $a_{ox}$ in purified RCs from $H. modesticaldum$ is a function of light, temperature and $O_2$. In the presence of $O_2$, we observe an immediate conversion of BChl $g$ to Chl $a_{ox}$ in the light, but there is a distinct lag in the absence of light. The length of the lag as well as the conversion process is temperature dependent. The Arrhenius behavior of the dark conversion process indicates that the HbRC is increasingly stable as the temperature is lowered, which is of practical importance for the handling of these preparations. At 9 °C, the duration of the slow kinetic phase before the relatively sharp transition to the fast conversion of BChl $g$ to Chl $a_{ox}$ is about 12 hours. When the sample is kept on ice, the duration of the slow kinetic phase is on the order of days. Increasing the temperature to 32 °C results in a rapid conversion of over 90% of the BChl $g$ to Chl $a_{ox}$ within 10 min. Hence, the conversion of BChl $g$ to Chl $a_{ox}$ can be precisely controlled with the use of light, oxygen and temperature. Significantly, at 0 °C and in darkness, the conversion was so slow that we were able to handle the RCs for 12 hours in weak green light in air without detectable conversion of BChl $g$. We can surmise that the activation energies observed for the conversion of BChl $g$ reflect a rate-limiting barrier. The fact that there is a lag phase suggests a multistep mechanism involving an intermediate that must
accumulate to a critical concentration before the conversion can occur to any large extent. This is supported by the observation that the conversion is strongly inhibited by menadione, which implies that it hinders the accumulation of the intermediate. The fact that isosbestic points are observed in the absorption spectra implies that the proposed intermediate does not absorb in the UV-Visible region. It is likely that several different reactions occur either simultaneously or in sequence since the several different oxidized forms of Chl $a_{ox}$ are observed. A small amount of genuine Chl $a_F$ was also found, but it was likely formed by exposure to acid in the post-treatment step prior to HPLC analysis. The mechanism by which the 1,3-hydride shift occurs is not known, however, it is thermally forbidden according to the Woodward-Hoffmann rules$^{34}$. Thus, in solution it requires acid$^{24}$. Our data show that it occurs in combination with oxidative addition of hydroxyl groups and that subsequent dehydration of the dihydroxyl adduct may also occur.

The data also indicate that as BChl $g$ is converted to Chl $a_{ox}$, the homodimeric BChl $g'/BChl\ g'$ special pair is converted at the same rate as the bulk BChl $g$ cofactors, which implies the presence of an intermediate heterodimeric Chl $a'_{ox}/BChl\ g'$ special pair prior to the final homodimeric Chl $a'_{ox}/Chl\ a'_{ox}$ special pair. The flavodoxin reduction data, the $P_{800}$
photobleaching, the appearance of biphasic charge recombination kinetics and changes in the line-width of the trEPR spectra all suggest that the heterodimeric special pair may be functionally active. The significance of this finding is that it implies that three different forms of the special pair can be generated and studied within the same protein matrix. The flavodoxin assay indicates that RCs that contain BChl \( g'\)/BChl \( g' \) and Chl \( a'_{ox}/BChl \) \( g' \) special pairs are functional in carrying out electron transfer, whereas RCs that contain the Chl \( a'_{ox}/Chl \) \( a'_{ox} \) special pair are not. This implies that the conversion does not completely impair the function of the RCs until both BChl \( g' \) molecules of the special pair have been converted.

Using a model in which only conversion of the special pair is considered and it is assumed that only Chl \( a'_{ox}/Chl \) \( a'_{ox} \) special pairs are inactive, it can be shown that at as BChl \( g' \) is increasingly converted to Chl \( a'_{ox} \) the ratio of Chl \( a'_{ox}/BChl \) \( g' \) special pairs to BChl \( g'/BChl \) \( g' \) special pairs increases, however, this process occurs at the expense of total functional RCs. For example, at 70% conversion of BChl \( g \), the functional RCs consist of a 4.2:1 ratio of Chl \( a'_{ox}/BChl \) \( g' \) special pairs relative to BChl \( g'/BChl \) \( g' \) special pairs. Their sum represents 52% of the number of initially functional BChl \( g'/BChl \) \( g' \) special pairs. It is therefore statistically possible to obtain a majority population of HbRCs with heterodimeric special pairs by selective
pigment oxidation. The model, which fits the experimental data, assumes that the quantum yield of charge-separation is the same in RCs containing BChl \( g'/\text{BChl } g' \) and Chl \( a'_{\text{ox}}/\text{BChl } g' \) special pairs. This is in contrast to RCs of a \textit{Rhodobacter capsulatus} mutant, which incorporate a BPh/\text{BChl} heterodimer as the primary donor and show a roughly 50\% reduction in the quantum yield of electron transfer\textsuperscript{35}. However, the latter are type II RCs with unidirectional electron transfer, while HbRCs are homodimeric type I RCs, in which the electron transfer is expected to be bidirectional.

The samples that are expected to contain a majority of Chl \( a'_{\text{ox}}/\text{BChl } g' \) special pairs also show the largest relative amounts of the fast charge recombination phase and the species with a broader P\textsubscript{800+} line-width. All of these data are consistent with the presence of RCs that contain a population of functional Chl \( a'_{\text{ox}}/\text{BChl } g' \) special pairs. In contrast to purple bacterial reaction centers, shift of the absorption bands in HbRCs due to excitonic coupling are relatively small (~10 nm). Hence, bleaching of the proposed Chl \( a'/\text{BChl } g' \) special pairs is expected to occur near either at the absorbance wavelength of BChl \( g \) (800 nm) or Chl \( a_f \) (670 nm). The data in \textbf{Figure 4.5} all point toward bleaching occurring only at 800 nm. However, this evidence is mostly indirect and further studies are needed to determine more
conclusively whether heterodimer special pairs are present and where they absorb.

These findings may have physiological implications for the fitness and survivability of *H. modesticaldum* in its native environment. Heliobacteria are soil-dwelling organisms that exist primarily as terrestrial phototrophs rather than aquatic phototrophs and are thought to have evolved in a mutualistic relationship with plants, particularly rice. They are strict phototrophs, hence they use light as an energy source but rely on fixed carbon in the form of pyruvate, lactate and/or acetate supplied by a plant. In return, they fix nitrogen. The HbRC uses light to generate ATP by cyclic electron transfer, thereby supplying the energetic requirements required for nitrogen fixation. The environment of a germinating rice patty provides the anoxic conditions for BChl *g* to be retained in a stable form in the presence of light. However, were the heliobacteria to become transiently exposed to *O*₂, there would be a rapid conversion of BChl *g* to Chl *a*₁ox. *H. modesticaldum* is, moreover, a mild thermophile, growing optimally at 48 °C, hence the conversion would be especially rapid. However, given that the BChl *g’/BChl *g*’ and Chl *a’*₁ox/BChl *g*’ special pairs are both functional, the organism would be able to withstand 50% conversion of BChl *g* while still retaining 75% functional RCs. This might provide enough of an energetic margin for the
organism to repair the damage and survive in what would otherwise be an inhospitable environment.
4.6 References


4.7 Figures

**Figure 4.1.** Molecular structure of (A) BChl $g$, (B) Chl $a_F$ and (C) $8^1$-OH Chl $a_F$ according to the IUPAC numbering system.
Figure 4.2. Effect of light and O$_2$ on the conversion of BChl $g$ to a Chl $a_{ox}$ in isolated heliobacterial RCs at 298 K. The light intensity at the surface of the cuvette was 40 $\mu$mol m$^{-2}$ s$^{-1}$ and the dissolved O$_2$ concentration of the solution was 8.3 mg L$^{-1}$ (air saturated water at 298 K, corrected for barometric pressure).

(a) Spectra at 10 min intervals. (b) Time course of the decline in the amplitude of the $Q_y$ peak of BChl $g$ ($A_{788}/A_{788}(t=0)$, black squares) and the subsequent rise in the amplitude of the Chl $a_{ox}$ $Q_y$ peak ($A_{670}-A_{670}(t=0)$, red circles). (c) Decline in the amplitude of the $Q_y$ peak of BChl $g$ at various temperatures. The data was fit with an exponential decay function to obtain rate constants of $1.01 \times 10^{-4}$ s$^{-1}$ (297 K), $1.67 \times 10^{-4}$ s$^{-1}$ (304 K), $2.5 \times 10^{-4}$ s$^{-1}$ (309 K), $3.45 \times 10^{-4}$ s$^{-1}$ (314 K), and
6.25 \times 10^{-4} \text{ s}^{-1} (319 \text{ K}). (d) Arrhenius plot for the first-order rate constant of the conversion of BChl g to Chl $a_{\alpha x}$. 
**Figure 4.3.** Effect of O$_2$ on the conversion of BChl $g$ to Chl $a_{ox}$ in an isolated heliobacteria RC at 298 K in the dark. (a) Representative spectra taken over the course of 60 hours. (b) Time course of the decline in the amplitude of the $Q_y$ peak of BChl $g$ ($A_{788}/A_{788}(t=0)$, black squares) and the subsequent rise in the amplitude of the Chl $a_{ox}$ $Q_y$ peak ($A_{670} - A_{670}(t=0)$, red circles). (c) Fast phase of the decline in the amplitude of the $Q_y$ peak of BChl $g$ at various temperatures. For each temperature, the zero point on the time axis was chosen as the time at which the transition from the initial lag phase to the fast phase was deemed to be complete (Table 4.1). The initial absorbance of the samples was 0.85. The fitted
exponential rate constants are indicated in the legend. The data were fit with an exponential decay function to obtain rate constants of $1.885 \times 10^{-4} \text{ s}^{-1}$ (298 K), $3.109 \times 10^{-4} \text{ s}^{-1}$ (306 K), $4.543 \times 10^{-4} \text{ s}^{-1}$ (310 K) and $5.403 \times 10^{-4} \text{ s}^{-1}$ (314 K). (d) Arrhenius plot for the first-order rate constant of the conversion of BChl g to Chl $a_{ox}$.
**Figure 4.4.** HPLC and mass spectrometry analysis of RC pigments during O$_2$ exposure. (a) HPLC retention traces of RC pigments monitored at 410 nm during exposure to O$_2$. (b) Plot of the linear loss of BChl $g'$ as a function of total BChl $g$ conversion. All HPLC spectra were normalized by carotenoid content (Peak 8). (c) UV-visible spectra of each of the 8 peaks observed during O$_2$ mediated conversion. Assignments of pigments were determined by both the m/z values and UV-visible absorbance spectra for each peak.
Peak 7 represents the acid mediated isomerization of BChl $g$ under acidic conditions due to residual oxygen in either the extraction solution (0.1% formic acid/acetone) or the RC prep sample.
Figure 4.5. \(P_{800}^*\) photooxidation and flavodoxin reduction as a function of BChl \(g\) conversion. (a) Relative flavodoxin reduction rate (open circles) and light-induced photobleaching of \(P_{800}\) (solid squares) taken at various states of the RC throughout exposure to light and \(O_2\). The predicted total population of primary donors composed of at least one BChl \(g'\) molecule (either BChl \(g'/BChl\ g'\) or BChl \(g'/Chl\ a'_{ox}\)) is shown as a dashed red line. (b) Light saturation curves for flavodoxin reduction in the 0\% (black squares and line) and 70\% BChl \(g\) converted samples (red squares and line). The dotted line depicts the half-saturation values. The inset shows the double reciprocal plot of flavodoxin reduction versus light intensity. The difference in the y-
intercept for the two samples indicates the decrease in $V_{\text{max}}$ upon conversion whereas the shift in the x-intercept indicates an increase in the half-saturation value or $K_M$ (c) Content of primary donor populations BChl $g'/\text{BChl } g'$, BChl $g'/\text{Chl } a'_{\text{ox}}$, or Chl $a'_{\text{ox}}$/Chl $a'_{\text{ox}}$ as a function of BChl $g$ conversion (see text for details). (d) Difference spectra of as isolated HbRCs (black squares and solid line) and HbRCs with 89% of initial BChl $g$ converted to Chl $a_{\text{ox}}$ (open circles and dashed line).
Figure 4.6. Time-resolved field modulation-detected EPR studies of the P$_{800}^+$ radical during conversion of BChl $g$. (A) Charge recombination decay kinetics as a function of oxygen exposure. (B) Amplitudes of the kinetic phases of the charge recombination kinetics. Red circles: 4.2 ms phase; blue circles: 0.3 ms phase; black circles: total amplitude of both phases. (C) Amplitude of stable photo-accumulated P$_{800}^+$. 
Figure 4.7. Spin-polarized trEPR spectra of $P_{800}^+$ $F_X^-$ taken at X-band (A) and Q-band (B). The black traces are spectra recorded at 80 K after exposure to oxygen for the amount of time indicated next to each spectrum. The red traces are simulations described in the text.
Figure 4.8. (A) Spin-polarized triplet state spectra. The upper two spectra are experimental (black) and simulated (red) spectra before and after exposure to oxygen. The narrow emission/absorption pattern at the center of the spectra is due to the radical pair $P_{800}^+F^{-}$. The lower two spectra in panel A are simulations of spectra of the triplet states of BChl $g$ and Chl $a_{\alpha}$. In
both cases the triplet state is formed by intersystem crossing. (B) Relative amplitudes of the BChl $g$ and Chl $a_{ox}$ contributions to the triplet spectra. The red circles are the BChl $g$ contribution and the blue circles are the Chl $a_{ox}$ contribution.
### 4.8 Tables

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>Duration (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>273</td>
<td>&gt; 1500</td>
</tr>
<tr>
<td>282</td>
<td>720</td>
</tr>
<tr>
<td>298</td>
<td>245</td>
</tr>
<tr>
<td>306</td>
<td>110</td>
</tr>
<tr>
<td>310</td>
<td>66</td>
</tr>
<tr>
<td>313</td>
<td>54</td>
</tr>
</tbody>
</table>

Table 4.1. Duration of the slow kinetic phase of BChl g oxidation in the dark as a function of temperature
Chapter 5

Future Directions and Concluding Remarks
Due to their homodimeric nature, the reaction centers (RCs) of heliobacteria, green sulfur bacteria (GSB), and chloracidobacteria most likely resemble the common ancestor of all Type I RCs. This fact, coupled with the recent discovery of the oxygen tolerant RC from *C. thermophilum*, suggests study of these RCs will offer valuable insight into the evolution of photosynthesis. Despite this promise, the oxygen sensitivity of GSB and heliobacteria has limited the full description of their electron transfer (ET) processes. The work presented in this dissertation addresses these deficiencies through thermodynamic and kinetic characterization of the photochemical ET reactions of homodimeric RCs from both anaerobic and aerobic phototrophs.

The RC in heliobacteria lacks chlorosomes, can be separated from its associated cytochrome and ferredoxin proteins, and contains a bound antenna comprised solely of one bacteriochlorophyll (BChl), BChl *g*. The midpoint potentials of $A_0$ (−854 mV) and $F_X$ (−504 mV) are 100-200 mV more oxidizing than in Photosystem I (PSI). Despite these more oxidizing redox potentials, the free energy gap between $A_0$ and $F_X$ is suitably poised to allow reduction of ferredoxin while minimizing charge recombination with P*. In addition, the redox potentials of $F_A$ and $F_B$ in PshBI (−480 mV/−524 mV), PshBII (−453 mV/−527 mV), and HM1_2505 (−452 mV/−533 mV) are in good
agreement with those published for $F_A$ and $F_B$ in GSB ($-446$ mV/-501 mV)\(^2\). Moreover, the experimentally measured potential of $F_X$ in heliobacteria ($-504$ mV) is consistent with the estimate for $F_X$ in GSB by Setif et al. ($F_X \approx F_B \approx -501$ mV)\(^2,3\). However, it should be noted that the midpoint potential of $F_X$ in GSB still requires experimental confirmation. In light of the difficulty in measuring this value indirectly using $P_{840}^+$ as an observable at room temperature (due to forward ET from PscC), tracking the $P_{840}^+F_X^-$ radical pair (RP) by transient EPR (trEPR) and/or the $P_{840}^+$ cation radical using field-modulated EPR would be more appropriate strategies. In contrast, the midpoint potential of $F_X$ in *C. thermophillum* is $\sim80$ mV more reducing than in heliobacteria. However, this value may be influenced by the presence of the reduced $F_A$ and $F_B$ clusters in PscB. This possibility is supported by the observation that the slow kinetic phase, assigned to charge recombination from $F_B^-$, is only lost with prolonged exposure to 1 M sodium thiocyanate. Interestingly, a similar trend was observed for the midpoint potential of $F_X$ in PSI. In the presence of the $F_A$ and $F_B$ containing PsaC subunit, the midpoint potential of $F_X$ is $\sim50$ mV more negative than after removal of PsaC with urea\(^4\). If this precedent holds for CabRCs, the midpoint potential of $F_X$ in the absence of PscB may also be significantly more positive. To address this issue, $F_X$ will require titration in CabRCs lacking PscB. These experiments
would ideally be carried out on purified RCs so that the presence or absence of PscB can be tracked optically (using the slow charge recombination phase), magnetically (using the so far unidentified EPR resonances of F_A and F_B), and biochemically (using SDS-PAGE). Identification of the appropriate solution conditions (e.g. chaotrope, ionic strength) necessary to remove PscB without damaging F_X can be confirmed by screening samples for the presence of 3P_840 using trEPR. Finally, it would prudent to perform a new redox titration of F_X in the HbRC, in the presence of PshBI, to look for further evidence of this trend.

If ET in homodimeric RCs proceeds as P→A_0→F_X, as our EPR and optical data suggest, the edge-to-edge distances between ET cofactors may represent another deviation from the PSI paradigm. For example, application of the Moser-Dutton ‘ruler’ would predict the distance between A_0 and F_X in homodimeric RCs to be 4-5 Å shorter than between analogous cofactors in PSI. In the absence of an atomic resolution X-ray structure, this issue remains unresolved. However, the growing thermodynamic and kinetic similarities between RCs from heliobacteria, GSB, and chloracidobacteria suggest structural information obtained for any one RC would serve as a useful guide for the interpretation of the ET reactions of this class of phototrophs as a whole. Furthermore, when the role of the quinone is fully
understood, this structural data may offer valuable insight into the evolution of the ET chain of PSI, where the phylloquinone acceptors are obligatory for the reduction of the terminal iron-sulfur (FeS) clusters.

One of the long-standing challenges associated with working with the HbRC is its sensitivity to O$_2$\textsuperscript{6,7}. In the presence of O$_2$, BChl $g$ and BChl $g'$ are oxidized primarily to 8$^1$-OH Chl $a_F$. By decreasing the temperature and/or incident light intensity, this oxidation can be significantly retarded. If oxidation proceeds, losses in energy transfer from the antenna as well as in charge-separation are observed. Concomitantly, the line-width of the P$^*$F$_X$-RP broadens and the rate of charge recombination at low-temperature becomes biphasic. At room temperature, flavodoxin reduction and P$_{800}^*$ photobleaching suggest ET occurs beyond what is expected if oxidation of one of the BChl $g'$ pigments in the primary donor was sufficient to inactivate the RC. Together, these results imply that a heterodimeric primary donor of BChl $g'$ and Chl $a_{\text{ox}}$ remains functional in these oxidized RCs. This raises several important questions about the proposed heterodimeric donor. 1) Do the hyperfine interactions of the donor reflect a change in its chemical identity? How is the unpaired spin on P$^*$ (de)localized? 2) What is the new wavelength of maximal bleaching? 3) Has the midpoint potential changed? 4) What are the properties of $^3$P, measured optically, following chemical
reduction? Addressing these questions will require a wide range of experimental methodologies, including electron nuclear double resonance (ENDOR) to determine the identity and localization of the unpaired spin on the primary donor, broadband transient absorption spectroscopy to determine the new long-wavelength trap, electrochemical titration to measure the midpoint potential, and low-temperature ODMR spectroscopy to observe $^3P$. The results will undoubtedly paint a more complete picture of the photochemical function of this unique heterodimeric donor as well as potentially explain why the homodimeric Chl $a'_\text{ox}/\text{Chl } a'_\text{ox}$ donor is not active.

The controlled oxidation of BChl $g$ also provides an opportunity to examine energy transfer within the RC antenna. For example, although the appearance of $^3\text{Chl } a_\text{ox}$ and $^3\text{BChl } g$ (formed by intersystem crossing) increase as oxidation progresses, it is unknown whether or not Chl $a_\text{ox}$ maintains some capacity to transfer energy to the primary donor (albeit inefficiently). In addition, why are some remaining BChl $g$ pigments unable to transfer their energy to the primary donor? The former question can be addressed by selectively exciting Chl $a_\text{ox}$ while monitoring the amplitude of either the P$^+$$F^-$$\text{RP}$ or P$^+$ optical bleaching. The latter may be explained by the functional role that subpopulations of BChl $g$ play in energy transfer. Since low-temperature ODMR spectroscopy can differentiate between these different populations
based on their zero-field splitting parameters, their individual rates of oxidation could offer insight into energy migration within the antenna.

Overall, this dissertation provides evidence that the thermodynamics and kinetics of the ET pathways in both aerobic and anaerobic phototrophs are highly similar. Although the midpoint potentials of the ET acceptors are more oxidizing than in PSI, the terminal FeS clusters are still poised to reduce ferredoxins. These differences in redox potentials may reflect a fundamentally different ET pathway in homodimeric RCs, where ET proceeds from A_{0}^- directly to F_{x}. Finally, temperature, light, and oxygen, can be used to control the oxidation of BChl g. This offers a novel framework to explore the fundamental mechanisms of both charge-separation and energy transfer in a homodimeric RC.
References


VITA
Bryan H. Ferlez

Education
Ph.D. in Biochemistry and Molecular Biology 2016
The Pennsylvania State University, University Park PA
B.S. in Biochemistry and Molecular Biology 2008
The Pennsylvania State University, University Park PA

Publications

Honors and Awards
• DOW Sustainability Innovation Student Challenge Runner Up – $2,500 2012
• Graduate Student Research Exhibition 2nd Place, Engineering Division – $50 2013