STRUCTURAL AND FUNCTIONAL RELATIONSHIPS OF THE BACTERIOPHEOPHYTIN ENVIRONMENT IN A PHOTOSYNTHETIC REACTION CENTER PROTEIN

by

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ABSTRACT

The photosynthetic reaction center (RC) of the bacterium *Rhodobacter sphaeroides* is a pigment-protein complex in which electron transfer reactions efficiently capture solar energy in the form of charge separation. Embedded in this protein are two molecules of bacteriopheophytin (BPhe), one of which mediates electron transfer (H_A). Efficient electron transfer through H_A, as well as other cofactors of the RC, relies on both the spatial arrangement of the cofactors in the protein scaffold, as well as favorable interactions between the cofactors and their surrounding protein components. In this work, the interplay between the H_A cofactor and its protein environment are explored. Specifically, a leucine residue whose side chain projects orthogonally to the plane of the H_A macrocycle was changed by site-directed mutagenesis, creating a series of RC mutants with different side chains at this position (termed (M)214). The results derived from this work reveal that: (i) (M)214 plays a role in the pigment selectivity of the H_A binding pocket; (ii) the volume of the (M)214 side chain is important for fast forward electron transfer; and (iii) the (M)214 residue affects the configuration of a nearby bacteriochlorophyll pigment. In a subsequent chapter of this work, I describe the effects of H_A coordination state in RCs that assemble exclusively with zincbacteriochlorophyll in place of bacteriochlorophyll and BPhe. My results reveal that the coordination state of the $H_A Zn^{2+}$ metal plays a role in determining the yield of charge separation within the protein. In addition, it was found that these so-called Zn-RCs do not assemble with a full occupancy of a cofactor. Collectively, the results from this work serve to increase our basic understanding of the protein-cofactor interplay within photosynthetic systems.

PREFACE

Much of the work presented in this thesis is derived from publications of which I am either the first or second author. The text from Sections 2.9, 2.10, 2.11, 3.1, 3.3, 3.4, 3.5, 4.1, 4.2, and 4.3, including Table 3.1 and Figures 3.4, 3.5, 3.6, 3,7, 3.8, 3.9, 3.10, 4.1, and 4.2, have all been published in Saer, R.G., Hardjasa, A., Rosell, F.I., Mauk, A.G., Murphy, M.E., and Beatty, J.T., (2013) *Role of* Rhodobacter sphaeroides *photosynthetic reaction center residue M214 in the composition, absorbance properties, and conformations of the* H(A) *and* B(A) *cofactors.* Biochemistry, 52(13), 2206-17. F. I. Rosell obtained the low temperature spectra. A. Hardjasa crystallized the protein samples, produced Figures 3.9, 3.10, Appendix 1, and the text corresponding to sections 2.10 and 3.5. M. E. Murphy refined the crystallography dataset and assisted with sections 2.10 and 3.5. The low temperature spectroscopy equipment was the property of A. G. Mauk. I performed the remaining experiments, and conceived the design of the experimental workflow. All other figures and text in the above sections were written by me under the supervision of J. T. Beatty.

The time-resolved spectroscopy was performed in collaboration with the group of Dr. Neal Woodbury at Arizona State University. Sections 3.6, and 4.5, including Table 4.2 and Figures 3.11, 3.12, 3.13, 3.14, 4.3, and 4.4 were published in Pan, J., Saer, R. G., Lin, S., Guo, Z., Beatty, J.T., and Woodbury, N.W., (2013) *The protein environment of the bacteriopheophytin anion modulates charge separation and charge recombination in bacterial reaction centers.* J. Phys. Chem. B. 117(24), 7179-89. The text for sections 3.6 and 4.5 is my own, which is written based upon the results and discussion presented in Pan et al. The table and figures in the above publication are the work of J. Pan, and reproduced with permission in this thesis. J. Pan assembled the time-resolved spectrometer acquired the data. During this period, I visited the group of Dr. Woodbury to learn this spectroscopic technique, including the analysis of the obtained datasets, which were also performed by J. Pan. Experimental design was the work of Drs. J. Pan, S. Lin, N. W. Woodbury, J.T. Beatty, and I. All the protein mutants were created by me.

The text from Sections 2.12, 3.9, 4.6, 4.7, and 4.8, including Tables 3.3 and 3.4, as well as Figures 3.18, 3.19, 3.20, 3.21, and 3.22, are published in Saer, R.G., Pan, J., Hardjasa, A., Lin, S., Rosell, F. I., Mauk, A. G., Woodbury, N. W., Murphy, M. E., and Beatty, J. T. (2013) *Structural and kinetic properties of* Rhodobacter sphaeroides *photosynthetic reaction centers containing exclusively Zn-coordinated bacteriochlorophyll as chlorin cofactors.* BBA: Bioenergetics. (Dec 5). In this work, J. Pan performed the time-resolved spectroscopy, A. Hardjasa, crystallized the protein, M. E. Murphy performed the crystallographic data refinement, created Table 3.3 and Figure 3.9.3, as well as Appendix 2. F.I. Rosell obtained the low temperature spectroscopy data. The low temperature spectrometer was the property of A. G. Mauk. The experimental design was conceived by Drs. S. Lin, N.W. Woodbury, J. T. Beatty, and I. The protein material for this work was created by me. All other figures and tables, as well as the manuscript text, were produced by me.

The recipe of the RLB culture media (Section 2.1) is published in Jun, D., Saer, R.G., Madden, J.D., and Beatty, J.T. (2013) *Use of new strains of* Rhodobacter sphaeroides *and a modified simple culture medium to increase yield and facilitate purification of the reaction centre*. Photosynth. Res. (Jun 14). None of the experiments presented in Jun et al. are included in this thesis. A description of the plasmid pRS1 (Section 2.6) and its uses as an expression system for the *R. sphaeroides* photosynthetic reaction center has been published in Jaschke, P.R., Saer, R.G., Noll, S., and Beatty, J.T. (2011) *Modification of the genome of* Rhodobacter sphaeroides *and construction of synthetic operons*. Methods Enzymol. 497, 519-38. No text or figures in this thesis are derived from the above publication.

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LIST OF ABBREVIATIONS

β mutant	A mutant reaction center that assembles with
	BChl in place of a BPhe
μE	Microeinstein
ADP	Adenosine 5' diphosphate
Ap ^R	Amplicillin resistance
ATP	Adenosine 5' triphosphate
bchD	Gene encoding the delta subunit of the
	magnesium chelatase enzyme
BChl	Bacteriochlorophyll
BLAST	Basic local alignment search tool
Вр	Base pair
BPhe	Bacteriopheophytin
B _x	High energy absorption band of chlorins. Its
	absorption overlaps with the B_y band and
	collectively, these bands are called the Soret
	band
By	High energy absorption band of chlorins. Its
	absorption overlaps with the B_x band and
	collectively, these bands are called the Soret
	band
Chl	Chlorophyll
DEAE	Diethyl amino ethyl
DNA	Deoxyribonucleic acid
DNase A	Deoxyribonuclease A
DOXP	1-deoxy-D-xylulose-5-phosphate
E	Escherichia
EADS	Evolution-associated difference spectrum
EDTA	Ethylenediaminetetraacetc acid
FFT	Fast Fourier transform

FPLC	Fast protein liquid chromatography
HPLC	High pressure liquid chromatography
ICM	Intracytoplasmic membrane
IR	Infrared
Kb	kilobase
LDAO	Lauryldimethylamine-n-oxide
LH	Light Harvesting
LH1	Light harvesting 1
LH2	Light harvesting 2
mRNA	Messenger ribonucleic acid
NADP	Nicotinamide adenine dinucleotide
	phosphate
NTA	Nitrilotriacetic acid
PCR	Polymerase chain reaction
PDB	Protein data bank
Phe	Pheophytin
Puc	Operon encoding the LH2 complex proteins
Puf	Operon encoding the RC M/L and LH 1
	complex proteins
puhA	Gene encoding the RC H protein
Qx	The intermediate energy absorption band for
	chlorins in the visible region of the
	electromagnetic spectrum
Qy	The low energy absorption band for chlorins
	in the visible to near-IR region of the
	electromagnetic spectrum
<i>R</i> .	Rhodobacter
RC	Reaction center
RPM	Revolutions per minute
SLIM	Site-directed ligation independent
	mutagenesis

TC ^R	Tetracycline resistance
Zn-BChl	Zinc-bacteriochlorophyll
Zn-RC	A RC containing Zn-BChl in place of BChl
	and BPhe
Zn-β-RC	The Zn-RC with the (M)L214H mutation

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1. INTRODUCTION

1.1 Definitions and types of photosynthesis. Photosynthesis is a fundamental biological process which has shaped the atmospheric composition of Earth, as well as provided the vast amounts of food and energy resources that our modern civilization relies upon. The word "photosynthesis" has its roots in 1893, when Charles Barnes sought a term to distinguish the assimilation of carbon dioxide in plants from the metabolic processes of animals (such as eating food). Before this, both the processes were united under the term "assimilation" (Gest 2005). Barnes proposed two alternative words for the "assimilation" of carbon, as performed by higher plants: photosyntax and photosynthesis. Although Barnes preferred the former term, the latter was more widely adopted by the rest of the scientific community, and thus became established in the literature. While the word photosynthesis has its historical roots in carbon assimilation, in the 1950s it was discovered that the true nature of a photosynthetic organism is the ability to conduct photophosphorylation: the production of ATP from light energy. More recently, Howard Gest proposed a revised definition of what constitutes a photosynthetic organism by suggesting that such an organism derives a "major fraction" of their energy for cellular syntheses from light (Gest 1993). The reason for this was to exclude several genera of so-called quasi-photosynthetic bacteria, which can produce photosynthetic pigments, yet require oxygen for growth using oxidative phosphorylation (respiration). Furthermore, this revised definition excludes bacteria which can generate ATP via lightdriven proton pumps called bacteriorhodopsins, as these bacteria are unable to support their own growth using these proton pumps as the sole source of ATP.

Nature was quick to exploit the immense and virtually inexhaustible source of solar energy (currently, $\sim 3 \ge 10^{24}$ J/year (Bolton and Hall 1979)) that bombards Earth on a daily basis, as evidenced by the proximity of the evolution of photosynthesis to the origin of life on our planet. Photosynthesis is thought to have originated between 3.7 and 2.4 billion years ago. Our planet is estimated to be 4.54 billion years old, with the earliest biomarkers of life appearing in banded iron formations in rocks dated to be 3.8 billion years old (Dalrymple 2001; Bjorn and Govindjee 2009; Dauphas et al. 2004). When coupled to the consumption of inorganic carbon from the oceans and atmosphere, photosynthesis allows our planet to sustain the 600-1000 gigatons of living biomass that exists today (Falkowski et al. 2000).

Photosynthesis has branched over evolutionary timescales into two major forms. Oxygenic photosynthesis is the form that is performed by plants, algae and cyanobacteria. These organisms are commonly grouped as photoautotrophs, because they can produce organic carbon compounds from inorganic carbon (*i.e.*, carbon dioxide). This form of photosynthesis, which is thought to be responsible for the oxygenation of Earth's atmosphere as early as 3 billion years ago (Crowe et al. 2013), utilizes two separate photosystems to catalyze the light-driven coupling of electron transfer to proton translocation across a membrane. The terminal electron acceptor is NADP⁺, a molecule whose reduced form, NADPH, is used in a variety of biochemical reactions in the cell as a source of reducing power. As its name states, this form of photosynthesis evolves oxygen from the splitting of water as a source of electrons. The produced NADPH is subsequently used in the Calvin– Benson-Bassham cycle to produce fixed (organic) carbon compounds, such as sugars, and potentially all major components of a cell, and storage compounds such as starch (Blankenship 2002).

In contrast to oxygenic photosynthesis, anoxygenic photosynthesis does not result in the production of oxygen. This form of photosynthesis is thought to have preceded the oxygenic form due to the relative simplicity of the photosynthetic machinery, as well as the presence of the pigment bacteriochlorophyll (BChl) a in these systems. BChl a was proposed to have been ancestral to chlorophyll (Chl) a (Xiong et al. 2000; Hohmann-Marriott and Blankenship 2011). Organisms that perform anoxygenic photosynthesis may be photoautotrophic, but many are photoheterotrophs and are capable of using organic carbon compounds as the source of cell carbon. In anoxygenic photosynthesis, the captured light energy is used to drive electron transfer coupled to proton translocation across the inner membrane of photosynthetic bacteria. The resultant proton gradient gives rise to a proton motive force that is used to generate ATP. Due to its relative simplicity, the anoxygenic photosynthetic machinery of photosynthetic bacteria has been widely studied in photosynthesis research to date, ranging from a purely fundamental understanding of the underlying mechanisms to applied research in light-driven energy generation for photovoltaic systems (Moser et al. 1992; Mahmoudzadeh et al. 2011). Furthermore, the close association of photosynthesis with the beginning of life on Earth makes studies of photosynthetic organisms useful for understanding how life began on this planet.

Bacterial photosynthesis is best understood in the purple bacteria, most of which are members of the α proteobacteria. These organisms have been the subject of a wealth of photosynthesis research in the decades since they were first characterized by Hans Molisch (Molisch 1907), and later in an extensive report by C. B. van Niel (van Niel 1944). Of these organisms, *Rhodobacter sphaeroides* is the most well-characterized in terms of the structure and function of the photosystem. In addition to being amenable to study using almost all

forms of spectroscopy, this organism features a sequenced (and recently revised with new annotations some errors corrected) genome (Mackenzie et al. 2001; Kontur et al. 2012), high resolution crystal structures of its photosynthetic apparatus (Allen et al. 1987b, a; Yeates et al. 1987), as well as a repertoire of genetic tools for both genomic and in *trans* manipulations. *R. sphaeroides* is also easily cultured, being capable of growing aerobically in darkness (a trait that some anoxygenic photosynthetic bacteria lack) and anaerobically in the absence illumination, as well as by use of photosynthesis.

1.2 Pigments of photosynthetic bacteria. The pigments utilized by photosynthetic bacteria to harvest light energy are of two main types, chlorins and carotenoids, and vary between different species. Representative structures of these pigments are shown in Figure 1.1.

In *R. sphaeroides*, BChl *a* is the most abundant pigment. This molecule is similar to plant Chl *a* in that it consists of a large tetrapyrrole macrocycle containing an additional "isocyclic" 5^{th} ring, as well as a hydrophobic phytyl tail that makes this pigment soluble in the cell membrane and a variety of organic solvents. Two modifications, a reduction of a double bond on ring B and the presence of a 3' acetyl group instead of a vinyl group on ring A, distinguish this pigment from the Chl *a* of higher plants (Scheer 2006). Such modifications result in a shifting of the pigment's absorption spectrum from the visible region (where Chl *a* absorbs) to the far red/near infrared region. This makes BChl *a* a useful pigment for photoheterotrophic bacteria, as it allows them to avoid competition (with plants and cyanobacteria) for photons by using wavelengths of light that are not absorbed by Chl-containing species such as plants and cyanobacteria. BChl *a* is functional as both a light-harvesting antenna pigment, as well as a redox-active cofactor in the capture of light energy.

The magnesium (Mg^{2+}) at the center of the all BChl macrocycles is bound to the four pyrrole nitrogen atoms from each surrounding ring. In addition, an exogenous fifth ligand must be present to stabilize this complex, as the Mg^{2+} of BChl is never found in a 4-coordinate state. In the photosynthetic pigment-protein complexes of *R. sphaeroides*, this ligand is almost always an axial histidine residue, although in other systems, other ligands such as asparagine, glutamine, water, certain organic solvents, and even other BChl molecules can provide a fifth coordinate (Heimdal et al. 2007; Evans and Katz 1975). Furthermore, mutagenic studies on BChl in protein complexes, in addition to studies of free BChl in organic solvents, have shown that it is possible for BChl to exist in a 6-coordinate state, with a second axial ligand coordinating the Mg^{2+} from the face of the macrocycle opposite to the fifth coordinate (Frolov et al. 2010; Evans and Katz 1975).

Bacteriopheophytin *a* (BPhe *a*) differs from BChl *a* by the absence of the central Mg^{2+} . The loss of this ion makes BPhe *a* more hydrophobic and electronegative molecule compared to BChl *a* (Noy et al. 1998). This gives the pigment properties relevant to electron transfer reactions in the capture of energy from sunlight. BPhe can be easily prepared by acid treatment of BChl, however the *in vivo* biosynthetic pathway to BPhe is not completely known. Enzymatic studies on the late stages of BChl biosynthesis indicate that BPhe is synthesized from BChl, because Mg^{2+} -lacking analogues of BChl synthesis intermediates are not good substrates for their respective enzymes in the BChl biosynthetic pathway (Oster et al. 1997).

Carotenoids are a class of pigments characterized by a long, conjugated series of carbon-carbon bonds with a variety of different functional groups located on the ends of the molecule. These pigments are all synthesized from the 5-carbon precursor molecule isoprene, which can be formed via the mevalonate pathway, or the more recently discovered 1-deoxy-D-xylulose-5-phosphate, or DOXP pathway. Both pathways seem to be employed by purple bacteria, albeit without any sort of obvious taxonomic pattern (Rohmer 1999). In bacterial photosynthesis, carotenoids play an important role in light-harvesting, as well as protection from photo-oxidative stress (Griffiths et al. 1955; Cogdell and Frank 1987). Most of the carotenoids produced by anoxygenic photoheterotrophic organisms differ from those of higher plants, algae, fungi, animals, and non-photosynthetic bacteria (Takaichi 2008). *R. sphaeroides* primarily synthesizes two carotenoids, sphaeroidene and sphaeroidenone, with sphaeroidene synthesized under anaerobic conditions. In the presence of oxygen, the enzyme sphaeroidene monooxygenase adds a keto group to the C2 carbon of sphaeroidene, generating sphaeroidenone (Takaichi 2004).



Figure 1.1. Chemical structures of the pigments from the photosynthetic apparatus of *R*. *sphaeroides*. Note the phytyl tails of BChl and BPhe are displayed as a separate molecule in the free alcohol form. The rings of the BChl and BPhe macrocycles are labeled according to the IUPAC convention.

1.3 The photosynthetic apparatus of *R. sphaeroides*. Despite the inherent photoactivity of photosynthetic pigments, their diverse functionality is ultimately achieved in the context of pigment molecules in an ordered structure. Nature accomplishes this by arranging the pigments in a protein scaffold. In *R. sphaeroides*, the photosynthetic machinery is composed of several proteins and pigment-protein complexes that work in concert to efficiently capture light energy in the visible and near infrared region, ultimately generating ATP. These proteins consist of two light-harvesting (LH) antenna complexes, a reaction center (RC) protein, a cytochrome *bc*₁ protein complex, and an F₀F₁ ATP synthase protein. All of these proteins are present as integral membrane proteins in the inner membrane, or water-soluble proteins in the periplasm. A schematic of these proteins and their functions is illustrated in Figure 1.2.



Figure 1.2. A schematic of photosynthesis in purple bacteria. Note: the RC/LH1 core complex is shown as a monomer for simplicity. Abbreviations: ADP = adenosine diphosphate; ATP = adenosine triphosphate; cyt = cytochrome; ox = oxidized; red = reduced; hv = light energy.

The LH complexes are pigment-protein complexes that capture light energy and funnel this excitation energy to the RC. In R. sphaeroides, two types of LH are present. Light-harvesting 2, or LH2, is the most abundant antenna structure during photosynthetic growth under low light intensity. LH2 consists of nine heterodimeric α/β protein subunits arranged in a membrane-spanning ring structure, with each subunit containing 3 molecules of BChl, and one carotenoid, for a total of 36 components (Sturgis and Niederman 2008; Strumpfer and Schulten 2009; Scheuring et al. 2003). This structure is not associated with the RC and exists as its own entity in the photosynthetic membrane. In fact, the regulation of LH2 expression at the genetic level differs from the RC and the light-harvesting 1 complex (LH1) and, the ratio of LH2 to LH1/RC in *R. sphaeroides* and other photosynthetic bacteria grown under different conditions is highly variable. Previous studies have shown that this variability may be attributed to both changes in light intensity, as well as the stage of acclimation from aerobic to photosynthetic growth. Specifically, the LH2 complex is maximally expressed (relative to LHI) under conditions of low light intensity, as well as during the later stages of acclimation to photosynthetic growth and/or O₂-limited chemotrophic growth in the dark (Woronowicz et al. 2011; Woronowicz et al. 2012). The BChls associated with LH2 absorb light maximally at wavelengths of 800 and 850 nm.

The LH1 complex is a larger antenna complex than LH2, although both LH complexes are multimeric ring structures composed of membrane-spanning α/β helical subunits. Unlike the LH2 complex, however, LH1 is genetically regulated in an identical manner to the RC because the genes coding for both of LH1 and the RC are part of the same *puf* operon (Choudhary and Kaplan 2000). The LH1 and RC complexes come together to form a "core complex", which in *R. sphaeroides* is dimeric in structure, with two RCs

partially encircled by 28 LH1 α/β polypeptide subunits and a small polypeptide called PufX (Qian et al. 2005; Qian et al. 2013). LH1 contains two molecules of BChl in every α/β subunit, which absorb infrared light maximally at 875 nm. The role of this complex is to capture light energy, as well as relay excitation energy captured by LH2 to the RC.

The core complex, in combination with LH2 constitute the vast majority of the photosynthetic machinery in *R. sphaeroides*. These complexes are located in spherical vesicles within the cell called intracytoplasmic membranes (ICMs), which are formed by invagination of the cytoplasmic membrane, perhaps due to electrostatic interactions of the LH2 and LH1 polypeptides (Chandler et al. 2009). These ICM vesicles were proposed to be either attached to the cytoplasmic membrane as buds, or "free floating" cytoplasmic entities released from the cytoplasmic membrane, functioning analogously to a eukaryotic organelle (Tucker et al. 2010).

Atomic force microscopy on ICMs revealed that these structures do not consist of randomly interspersed LH2 and core complexes, but rather clusters of core complexes appear to be arranged in stacks, surrounded by "lakes" of LH2 (Bahatyrova et al. 2004). To date, only the core complexes and LH2 have been resolved by imaging techniques, and thus the localization of the other proteins of the photosynthetic apparatus (the ATPase and the cytochrome bc_1 complex) remains unknown.

The process of photosynthesis may be thought of as initiating with the absorption of light energy, usually by a photoactive pigment in an LH complex. Absorption of light energy promotes the pigment systems in the LH complex to an excited state. This excitation energy is then transferred to the RC, which can store this energy in the form of charge separation.

This is an extremely important reaction as it marks the point at which the light energy is trapped by conversion to chemical energy. The *R. sphaeroides* RC converts energy conversion via a network of pigments that catalyze a series of redox reactions, embedded in the protein, ultimately leading to the reduction of a diffusible quinone that functions as a proton/electron shuttle. This quinone diffuses laterally in the ICM, and docks with the cytochrome b/c_1 complex. The role of this complex is twofold. First, it oxidizes the quinol molecule back to a quinone, translocating the released protons from the cytoplasm into the periplasmic space (or the interior of the ICM in the case of a free floating vesicle). This accumulation of protons in the periplasm allows the ATPase enzyme to generate ATP from ADP, by utilizing the potential energy of the generated ion gradient. The second function of the cytochrome b/c_1 complex is to reduce a periplasmic c-type cytochrome (cytochrome c_2 in *R. sphaeroides*). This cytochrome serves to reduce the RC, which has become oxidized from the conversion of light energy to chemical energy. In addition, the cytochrome b/c_1 complex, in a process called the Q cycle, reduces a quinone to additionally contribute to uptake of protons from the cytoplasm (Fig. 1.2).

1.4 Reaction center types, structure and function. RCs can be grouped into two main types: type I and type II. Type I RCs are easily identified by the presence of three redox-active iron-sulfur clusters of the [4Fe-4S] type, which act as electron transfer intermediates. The protein component of these RCs may be either heterodimeric, as in photosystem I from plants and cyanobacteria, or homodimeric, as in the RCs from the *Heliobacteriaceae*, *Chlorobiaceae*, and *candidatus Chloroacidobacterium thermophilum* (Liebl et al. 1993; Buttner et al. 1992; Bryant et al. 2007). The role of these RCs is to generate a strong

reductant capable of reducing NADP⁺ to NADPH. Because of this, they operate at very negative redox potentials (~ -1200 to -540 mV for photosystem I (Brettel 1997)).

Type II RCs are heterodimeric in structure and lack an iron-sulfur center. These RCs are found in cyanobacteria and higher plants (photosystem II is a type II RC), as well as in all purple photosynthetic bacteria. Instead of Fe-S clusters, type II RCs contain quinones as electron transfer intermediates. Since quinones are two-electron shuttles, a type II RC undergoes two turnovers before the quinone is fully reduced into a quinol state, at which point this molecule diffuses laterally through the membrane, away from the RC. Type II RCs generally operate at higher redox potentials than type I RCs (~ -580 to -80 mV for photosystem II, and ~ -1000 to ~ -80 mV for the *R. sphaeroides* RC), which, in the case of photosystem II, also create a powerful oxidant capable of splitting a water molecule (in concert with the oxygen-evolving complex (Grabolle and Dau 2005; Blankenship 2002)). Also present in these RCs are (B)Phe molecules, which are electron transfer intermediates that apparently have been selected by nature to facilitate charge transfer between (B)Chls and quinones.

The first high-resolution 3-D structure of a membrane protein was of the type II RC from *Blastochloris viridis* (then *Rhodopseudomonas viridis*), and consisted of 4 proteins -- 3 RC proteins and a bound, tetraheme cytochrome (Deisenhofer et al. 1985). Subsequently the RC of *R. sphaeroides* was crystallized and the structure solved, showing that this complex differs from the *B. viridis* RC by being composed of only 3 protein subunits, homologues of the *B. viridis* RC proteins and termed L, M, and H (Allen et al. 1987b). Apart from the tetraheme cytochrome the two structures are similar, and predicted amino acid sequences of RC proteins from a wide variety of purple photosynthetic bacteria indicate little variation

from the *B. viridis/R. sphaeroides* paradigm. This view was confirmed by the crystal structure of the RC from the moderate thermophile *Thermochromatium tepidum* (Nogi et al. 2000).

The L and M proteins are structurally symmetrical, sharing an axis of symmetry orthogonal to the plane of the membrane bilayer, and contain 5 transmembrane helices that house the cofactors buried inside. The cofactors consist of two excitonically coupled BChls (PA and PB; together, often called P), two accessory BChls (BA and BB), two molecules of BPhe (H_A and H_B), a non-heme Fe²⁺ ion (Boso et al. 1981), and two molecules of ubiquinone 10 (Q_A and Q_B). The overall structural arrangement of the RC is shown in Figure 1.3. The pigments of the RC are organized into two structurally symmetrical, but functionally asymmetrical branches, termed the A (active) and B (inactive) branches. The P BChls are located on the periplasmic side of the protein, and the quinones are located on the cytoplasmic side. Between P and QA, BA and HA serve as electron transfer mediators that also assist in extending the longevity of the charge-separated state, by creating a large physical distance between oxidized P and reduced QA. These cofactors are arranged such that when P is promoted to an excited state, electron transfer to an accessory pigment is the most rapid decay mechanism. The electron transfer cascade, as well as the approximate electron transfer rates, are shown in Figure 1.4. Ultrafast visible and IR spectroscopic studies of the RC indicate that the primary electron acceptor is B_A , which is reduced on a time scale of ~3 ps (Khatypov et al. 2012; Pawlowicz et al. 2008). This $P^+B_A^-$ radical then reduces H_A within 1 ps to generate the $P^+H_A^-$ state. The H_A^- radical anion reduces Q_A in ~200 ps followed by electron transfer to the Q_B quinone in a reaction (~200-300 µs) that is coupled to protonation of Q_B to produce an electronically neutral ubisemiquinone radical (Paddock et al. 2003). The

non-heme Fe²⁺ ion is located between the two quinones (not shown in Figure 1.3), and is presumed to play a role in mediating electron transfer from H_A to Q_A by tuning the electronic properties of the quinone (Ishikita and Knapp 2006; Kirmaier et al. 1986). A mobile cytochrome *c* reduces the oxidized P⁺, and the RC is set for a second turnover in which the Q_B ubisemiquinone is fully reduced to a quinol. This quinol is released from the RC and is oxidized at the cytochrome b/c_1 complex.



Figure 1.3. The three dimensional structure of the RC from PDB 2J8C. Left: The protein component surrounding the photoactive pigment cofactors. The L subunit is colored red, the M subunit is colored gray, and the H subunit is colored orange. Right: The embedded cofactors shown with the protein component removed. The two Q_B molecules present in the figure represent the different binding states of Q_B in the RC. The black circle denotes the non-heme iron atom. The shorter Q_B tail (relative to Q_A) is a result of this specific RC being reconstituted at the Q_B site with ubiquinone-2.



Figure 1.4. Electron transfer time constants within the RC of *R. sphaeroides*. The grey arrows and numbers indicate the direction and time constants of the back reaction. The cofactors that are not directly involved in electron transfer are not shown. Also missing from this diagram is the non-heme iron.

In experiments intended to elucidate the structure-function relationship of the RC, this pigment-protein complex has been subject to a vast degree of mutagenic and spectroscopic analysis, leading to the understanding that this protein exhibits a remarkable tolerance to changes in both the protein and pigment composition (Jones 2008). A key discovery in RC research was the ability to alter the pigment composition of the RC by the use of site-directed mutagenesis. Examples include the swapping of photoactive BPhe with BChl, in the H_A binding pocket, as well as the reverse exchange in both the P and B_A pockets (Bylina and Youvan 1988; Kirmaier et al. 1988; Nabedryk et al. 2000; Katilius et al. 2004; Kirmaier et al. 1991; Heller et al. 1995). RCs containing a molecule of BChl in place of BPhe at H_A are called "beta" mutants, and the H_A BChl molecule is commonly referred to as the " β " pigment. In addition to mutagenesis, the pigments of the RC can also be exchanged through biochemical means, allowing for the incorporation of synthetic or chemically modified chromophores into the RC (Scheer and Hartwich 1995). Such experiments have provided insights into the nature of biological electron transfer, as well as an understanding of the requirements of pigment binding to the protein scaffold.

1.5 Interactions between the chlorins and the protein component of the RC. The

crystal structure of the *R. sphaeroides* RC coupled with site-directed mutagenesis experiments have greatly contributed to our understanding of the interactions present between the photoactive cofactors and the protein component. Several pigment-protein interactions assist in fixing the pigments to the protein matrix, as well as tuning the electronic properties of the photoactive pigments in the RC. Figure 1.5 shows the known binding interactions of the BChl dimer (P) with the surrounding protein, to give an example of the different kinds of protein-cofactor interactions available in the RC. The P BChls are oriented

nearly perpendicular to the plane of the membrane, with their A rings overlapping. The magnesium ions of the P_A and P_B BChls are coordinated by histidine residues (L)173 and (M)202, respectively. An additional hydrogen bond between the ring A acetyl group of P_A and His (L)168 serves to raise the midpoint potential of the dimer for optimal electron transfer (Mattioli et al. 1994; Lin et al. 1994). The B_A monomer BChl contains no such protein-macrocycle hydrogen bonding, however this BChl Mg²⁺ ion is pentacoordinated by His (L)153 (Yeates et al. 1988). The interaction between B_A and His (L)153 is shown in Figure 1.6A.

The H_A cofactor, unlike the previously mentioned BChls, contains no axial histidine ligand, and no Mg^{2+} is present in the center of the macrocycle. A leucine residue at position (M)214 resides in an analogous axial position, however (Figure 1.6B). A nearby glutamate residue, Glu (L)104, forms a hydrogen bond with the keto carbonyl of ring E (Figure 1.6B), giving rise to a change in the absorbance properties of this cofactor relative to H_B (Yeates et al. 1988). The presence of a BPhe molecule at H_A , in addition to the hydrogen bond by Glu (L)104, ensures a high yield of the $P^+Q_A^-$ state during electron transfer. Yields as low as 27% have been reported in a mutant RC in which Leu (M)214 is changed to histidine (resulting in a molecule of BChl at H_A) and Glu (L)104 is changed to valine (removing the hydrogen bond from the ring E carbonyl group of H_A (Kirmaier et al. 1995b)).

1.6 Analysis of RCs (RC pigments) by optical absorption spectroscopy. Optical

absorption spectroscopy has been an invaluable tool in the study of all photosynthetic systems. The photoactive nature of photosynthetic pigments gives them characteristic spectra which reveal a wealth of information about the pigment's properties in a non-destructive manner. The use of absorption spectroscopy in the study of bacterial photosynthesis dates
back to the middle of the twentieth century, when Duysens began to characterize purple bacteria using the techniques of absorption and fluorescence spectroscopy (Duysens 1951). Interactions between pigments and other molecules (such as solvents, other pigments, polypeptides, *etc.*) give rise to changes, or shifts, in their absorption spectrum. This is especially evident in the light-harvesting complexes of *R. sphaeroides*, where the absorption bands of BChl are shifted over 100 nm to the red as a result of their spatial arrangement.



Figure 1.5. Bonding interactions of the BChl dimer with the surrounding protein. Histidines (M)202 and (L)173 act as fifth coordinates to the P_A and P_B , respectively, while his (L)168 donates a hydrogen bond to the ring A acetyl group of P_A . Ligating nitrogen atoms on histidines are shown in blue. Histidine residues are otherwise colored red. Carbon and oxygen atoms on the BChls are colored green and red, respectively. The yellow dashed lines denote ligation between the BChl nitrogen atoms and the central Mg^{2+} (green pluses). The red dashed lines denote ligation between histidine nitrogen atoms and the BChl Mg^{2+} or carbonyl oxygen atoms. RC structural data was obtained from PDB 2J8C (Koepke et al. 2007).



Figure 1.6. Bonding interactions of the B_A and H_A cofactors with the surrounding protein. (A) Histidine (L)153 (red) acts as the fifth ligand to the Mg^{2+} of B_A through one of its nitrogen atoms (blue). (B) At H_A , Glu (L)104 (blue) donates a hydrogen bond to the carbonyl group on ring E of the HA macrocycle. Leu (M)214 (yellow) projects towards the center of the H_A macrocycle. The dashed lines denote ligation or hydrogen bonding interactions. RC structural data was obtained from PDB file 2J8C (Koepke et al. 2007).

All Chls and BChls contain four principle absorption components which represent the physically allowed $\pi \rightarrow \pi^*$ transitions that give rise to absorption bands in the visible and near IR region. The transitions are grouped along two nearly perpendicular axes that run along the plane of the chlorin macrocycle. These axes are denoted X (running from ring D to B), and Y (running from ring A to C), and are displayed in Figure 1.7. The two transitions for each axis are denoted B and Q, which carry the axis label as a subscript. The strongerabsorbing B_x and B_y bands normally overlap in chlorin type of pigments, and thus are collectively referred to as the Soret band. For BChl a, this is a large heterogeneous absorption band with a maximum around 360 nm. The other two transitions, Q_x and Q_y, give rise to longer wavelength absorption bands at 590 nm and 770 nm, respectively. The Q_x and Qy transitions have been especially useful in characterizing the environment of BChls because of their sensitivities to the surrounding environment. The RC is a case in point, as the Q_x and Q_y bands from the absorption spectrum of the RC can (almost) be resolved for each individual chlorin, at cryogenic temperatures where the absorption bands become sharper.

The absorption spectrum of the *R. sphaeroides* WT RC at 10 K is shown in Figure 1.8. This spectrum contains seven characteristic peaks (as well as shoulders) in the 250 to 950 nm range. Like all proteins, the RC has a strong absorbance band at 280 nm, which mainly represents contributions from aromatic amino acids, especially tryptophan, which has the strongest absorbance. The Soret band of all chlorins present (BChl and BPhe) is located ~80 nm to the red of the protein band. Most spectroscopic analysis of RCs and the corresponding literature, however, focus on the Q_x and Q_y transitions. The Q_x transitions of the *R. sphaeroides* RC at 10 K are represented by three resolved peaks. The two bands at

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~535 and ~545 nm represent contributions from the Q_X transition of H_B and H_A , respectively. The overlapping peaks at ~590 to 605 nm represent contributions from all BChls, with the 590 nm peak assigned to the $B_{A,B}$ BChls, whereas the 605 nm shoulder is assigned to a combination of B_B and $P_{A,B}$ (Shochat et al. 1994; Kirmaier et al. 1985). The carotenoid sphaeroidenone (the predominant carotenoid when cultures grow in the presence of O_2) also absorbs in this region of the spectrum. The absorbance of this pigment is broadband and ill-defined in the region from approximately 500 nm to 650 nm.



Figure 1.7. The two molecular axes of BChl *a*. The dashed grey arrows depict the X and Y axes of the BChl molecule, respectively (Scheer 2006). Note: the positioning of electronic transition dipoles do not run exactly along the molecular axes because the angle of the transition dipole relative to the molecular axis is sensitive to the surrounding environment.

The Q_y transition region contains three characteristic peaks, all in the far red to near IR region of the spectrum. The 760 nm peak is composed of contributions from H_{A,B}, with the H_B peak on the blue side and the H_A peak on the red side. The B_{A,B} accessory BChls contribute to the large, overlapping ~ 802 nm peaks, with the red shoulder representative of B_B. Finally, the peak at 890 nm is assigned to P_{A,B}. The position of this peak is temperature-sensitive, and absorbs nominally at 865 nm when an absorption spectrum is recorded at room temperature. For this reason, P_{A,B} is frequently referred to as P865.



Figure 1.8. Ground state absorption spectrum of the wild type *R. sphaeroides* RC taken at 10 K. The labels in **bold** indicate the transition dipole of the photoactive pigments that give rise to the absorbance peaks. Terms that are not in bold indicate the specific cofactors that contribute to the amplitude of the peak. Assignments of peaks and shoulders were obtained from Jones and Kirmaier *et al* (Jones 2009; Kirmaier et al. 1985).

1.7 The protein environment around H_A, and the origin of BPhe. As mentioned in section 1.5, the protein backbone of the RC is responsible for tuning the electronic properties of the embedded pigments, as well as providing a fifth coordinate to the Mg²⁺ central ion of the BChls. In addition, the protein, in combination with the long hydrophobic tails of the pigments, serve to create a well-insulated environment in which electrons tunnel from pigment to pigment with little risk of a side reaction that would result in a decrease in the efficiency of energy transduction. Studies aimed at understanding this structure-function relationship between the pigments and the protein component are useful in developing a knowledge base on biological electron transfer reactions. Such reactions could prove useful in future engineering applications that capitalize on electron transfer reactions using biological, as opposed to synthetic, systems. In addition, an understanding of the mechanisms governing biological electron transfer within, and to and from different types of RCs provides insight into the evolution of such systems. Because an attempt to obtain a comprehensive understanding of all electron transfer reactions in the RC would be beyond the scope of a single PhD thesis, I focus on the electron transfer events which involve the BPhe cofactor.

The use of (B)Phe in native type II RCs is critical to ensure a successful electron transfer event to a quinone molecule of much lower free energy. The role of this molecule in the electron transfer cascade is twofold. First, it creates an anion radical intermediate that is physically further away from the oxidized donor molecule (P865), which greatly decreases the rate of the back reaction. Second, the (B)Phe is energetically distant from the (B)Chl monomer (B_A in *R. sphaeroides*), such that the (B)Chl and (B)Phe anionic states do not exist in equilibrium, ensuring that the large distance between the oxidized donor and its electron is

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maintained. In the *R. sphaeroides* RC, the lack of an axial ligand in the H_{A,B} pockets is crucial to the incorporation of this pigment into the RC. Instead of a ligand, a Leu residue is positioned axial to the BPhes in both the A and B branches at the (M)214 and (L)185 positions, respectively. The fact that BChl is found in the H_A pocket when (M)L214 is changed to His suggests that BPhe incorporates into the RC by one of two mechanisms: (i) the axial Leu residue contributes to a BPhe binding pocket selective for an already synthesized molecule of BPhe; or (ii) the axial Leu residue physically assists in the dechelation of Mg²⁺ from a BChl molecule, perhaps during RC assembly, resulting in BPhe. It is difficult to distinguish between these two possibilities for several reasons. The first is that, apart from the RC itself, BPhe is not detectable in the chromatophore membrane. Second, no Mg-dechelatase enzyme has been discovered in purple bacteria to date, although a heat-stable Mg-dechelating substance has been purified in senescent plant leaves, in addition to a Mgdechelation activity by the magnesium chelatase enzyme itself during the greening of plant leaves (Shioi et al. 1996; Ignatov and Litvin 1994).

It was thought that RC-mediated dechelation could occur in one of two stages of RC complex assembly, depicted in Figure 1.9. The first is during the assembly of the apocomplex, where (B)Chls are thought to initially bind the protein and, in studies conducted on plant photosystems I and II, assist in the proper folding of the protein (Mullet et al. 1990; Eichacker et al. 1996; Muller and Eichacker 1999). In purple bacteria, it is not known how the photosynthetic machinery is translocated into the bacterial inner membrane, however evidence from cyanobacterial photosystems reveals the requirement for the SecYEG translocation complex, in addition to a protein called YidC (Sobotka 2013). Assuming binding of (B)Chl to the protein at this stage of protein folding, prior to complex assembly, the Mg^{2+} ion may depart from the (B)Chl macrocycle unless an axial ligand is present. In the second stage of complex assembly, the independently stable transmembrane domains of the RC are formed, and the final structure of the protein begins to take shape via interactions between these helices in combination with the exclusion of lipids between domains (Popot and Engelman 2000). At this point in assembly, the bulky Leu side chains found orthogonal to the macrocycle plane of (B)Phes in the RC may act as a steric obstruction to the Mg^{2+} ion, resulting in its removal from the (B)Chl molecule.



Figure 1.9. Two stages of integral membrane protein development. In stage I, the nascent polypeptide forms stable, but disperse transmembrane domains in the lipid bilayer. In stage II, the domains begin to interact, forming the final protein structure with exclusion of the lipid component between helical domains. Figure reprinted with permission from Popot and Engelman (2000).

1.8 Thesis aims and approach. The goal of this work is multifaceted, in part because certain results of early experiments shed light on and led to other experiments. In one instance, I aimed to provide further insight into the origin of BPhe as it appears in the RC of R. sphaeroides, using a combination of in vivo, genetic, and biochemical techniques. The RC-mediated dechelation hypothesis (described above in Section 1.7) was tested. The work in this section focuses on the use of a series of nonpolar "cavity" mutants in which the Leu at position 214 on the M subunit of the RC was substituted with smaller, nonpolar moieties. The rationale behind such changes was that these moieties cannot act as a fifth coordinate to a BChl molecule, nor can they act as a steric impediment to the Mg²⁺ center of a BChl molecule during RC assembly. The binding criteria of H_A was further investigated using a series of mutant RCs in which polar amino acid residues (Gln, His, Asn) were introduced at this site. It was found that RCs with amide-containing side chains at position (M)214, were also capable of assembling with BChl at H_A. Because of this, I also examined the spectroscopic differences between these RCs, as the amide-containing mutants used an oxygen atom for ligation (compared to a nitrogen in and Leu -> His mutant), and the BChlligand distances differed between the amide-containing mutants. The RCs described above were studied in the context of whole cells during periods of photosynthetic anaerobic growth, as well as studies of isolated protein using absorption spectroscopy, biochemical pigment substitution methods, and X-ray crystallography.

The results derived from spectroscopic analysis of RCs containing small, nonpolar moieties at (M)214 led me to pursue a functional investigation of these proteins. In this arm of my research, I collaborated to investigate the performance of these RCs through ultrafast spectroscopic techniques. The results obtained allowed me to propose explanations of

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changes in electron transfer rates due to structural perturbations that resulted from engineered mutations at (M)214. These data bring new insight into the means by which RCs generate charge separation with extremely high efficiency.

In a subsequent section of my research, I employed the same techniques used to study the small, nonpolar mutant RC series on a different RC that assembles entirely with Zn-BChl in place of both BChl and BPhe. These RCs were originally characterized in a Mg-chelatase (*bchD*) mutant of *R. sphaeroides* (Jaschke and Beatty 2007). Our ultrafast spectroscopic studies on these proteins have enabled the discrimination of the effects on electron transfer that can be ascribed to the type of central metal ion, versus the presence of a fifth coordinate at H_A. The crystal structures of these RCs are also reported, revealing the structural consequences of assembling RCs of wild type amino acid sequence but unnatural pigment composition.

Collectively, these data further the understanding of the structure-function relationship of photosynthetic RCs. The molecular mechanisms relating RC structure and electron transfer rates and pathways are extremely complex, and experiments such as the ones presented in this work may aid in the creation of more refined and accurate models to describe these phenomena. Finally, in the process of optimizing the preparation of sufficient material for these experiments, several new strains, plasmids, and protein isolation techniques were developed, which may aid the scientific community in research on this remarkable organism and its iconic photosystem.

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2. MATERIALS AND METHODS

2.1 Bacterial strains and plasmids. Table 2.1 lists the different strains of *Escherichia coli* and *R. sphaeroides* used in this thesis. *E. coli* Strains DH5α and DH10B were used for cloning. *E. coli* strain HB101 (pRK2013) was used as a helper strain for mobilization of plasmids from *E. coli* into *R. sphaeroides* via tri-parental conjugation (Ditta et al. 1985).

R. sphaeroides strain Δ RCLH was used as a background strain for the *in trans* expression of photosynthetic RC-LH1 core complexes (Tehrani and Beatty 2004). This strain lacks the genes required for the synthesis of RC-LH1 core complexes, in addition to LH2. *R. sphaeroides* strain Δ *puhA* was used as a negative control for photoheterotrophic growth experiments (Chen et al. 1998).

For the growth of *E. coli*, cultures were grown aerobically on LB medium in glass test tubes at 37 °C. LB contains 1% tryptone, 0.5% yeast extract, and 1% NaCl per liter of media (Sambrook et al. 1989). For aerobic growth of *R. sphaeroides*, cultures were grown in sterile glass test tubes filled with RCV medium at 30 °C (Beatty and Gest 1981). RCV medium contains per liter: D,L-malic acid, 4 g; (NH₄)₂SO₄, 1 g; potassium phosphate buffer (pH 6.8), 10 mM; MgSO₄ · 7 H₂O, 200 mg; CaCl₂ · 2 H₂O, 75 mg; FeSO₄ · 7 H₂O, 12 mg; Na₂ EDTA, 20 mg; trace element solutions (containing, per 250 mL of dH₂O: H₃BO₃, 0.7 g; MnSO₄ · H₂O, 398 mg; Na₂MoO₄ · 2 H₂O, 188 mg; ZnSO₄ · 7 H₂O, 60 mg; Cu(NO₃)₂ · 3 H₂O, 10 mg), 1 mL; thiamine hydrochloride, 1 mg; biotin, 15 µg; niacin, 1 mg. For semi-aerobic growth, cultures were grown in the dark in Erlenmeyer flasks filled to 70% nominal capacity with RLB medium. RLB medium contains per liter: 10% tryptone, 5% yeast extract, and 0.23% NaCl, in addition to MgCl₂ and CaCl₂ at final concentrations of 810 µM and 510 µM, respectively (Jun et al. 2013). Semi-aerobically grown cells were shaken at 150 RPM, instead of the 200 RPM used for aerobic growth conditions. For anaerobic, photoheterotrophic growth of *R. sphaeroides*, cultures were grown in 16.5 mL screw cap tubes filled completely with RCV media. These tubes were incubated at 30 °C in a temperature-controlled aquarium and illuminated with an array of incandescent light bulbs.

For plasmid selection in *E. coli*, media was supplemented with antibiotics at the following final concentrations: tetracycline-HCl at 10 μ g/mL, kanamycin sulfate at 50 μ g/mL, ampicillin at 100 μ g/mL. For plasmid selection in *R. sphaeroides*, tetracycline was used at 2 μ g/mL final concentration.

Liquid culture densities of *R. sphaeroides* cells were measured with a Klett-Summerson photometric colorimeter equipped with a red filter (100 Klett units ~ 3×10^8 CFU mL⁻¹).

Strains	Relevant characteristics	Source or reference
E. coli		
DH5a	F^{-} , $\Delta(lacZY A - argF) deoR$, $recA1$, $endA1$	Invitrogen
DH10B	F ⁻ , mcrA, recA1, endA1, ara Δ 139, Δ lacX74	Invitrogen
HB101	pRK2013 contains RK2 transfer genes	(Ditta et al.
		1985)
R. sphaeroides		
ΔRCLH	RC ⁻ , LH1 ⁻ , LH2 ⁻	(Tehrani and
		Beatty 2004)
ΔpuhA	RC ⁻ , LH1 ⁺ , LH2 ⁺	(Chen et al.
		1998)
$\Delta puhA\Delta puc\Delta bchD$	RC ⁻ , LHI ⁺ , LH2 ⁻ , Mg Chelatase ⁻	(Jaschke and
		Beatty 2007)
$\Delta \text{RCLH} \Delta bchD$	RC ⁻ , LHI ⁻ , LH2 ⁻ , Mg Chelatase ⁻	(Neupane et
		al. 2012)
R. capsulatus		
$\Delta g ta I \Delta 1081$	C16 homoserine lactone ⁻ , capsular polysaccharide ⁻	(Brimacombe
		et al. 2013)

Table 2.1 Bacterial Strains

The plasmids used in this work are listed in table 2.2. Plasmids created in this work are described in detail following this table.

Plasmid	Relevant characteristics	Source or reference
pATP19P	Broad host range expression vector	(Tehrani and
	containing the hypoxia-inducible puc	Beatty 2004)
	promoter, Tc ^r	
pTZ18U	Cloning vector, Ap ^r	Pharmacia
pTZ18U::puhA	pTZ18U containing the <i>RC-H</i> gene from <i>R</i> .	(Chen et al.
	sphaeroides as a 1.34 Kb BamH I fragment	1998)
pTZ18U::puhA T	pTZ18U:: <i>puhA</i> with a putative Rho-	This study
	independent transcriptional terminator	
	removed between the <i>puhA</i> stop codon and	
	the BamH I restriction site	
pZT18U::6 his <i>puhA</i> T ⁻	pTZ18U:: <i>puhA</i> T ⁻ containing a c-terminal	This study
	6x histidine tag on the <i>puhA</i> gene, in	
	addition to the changing of the GCC codon	
	immediately 5' of the tag to a GTG codon,	
	thereby introducing an ApaL I site	
pZT18U::6 his <i>puhA</i> T⁻	pZT18U::6 his <i>puhA</i> T ⁻ with the <i>Bam</i> H I	This study
3'sac	site downstream of the <i>puhA</i> gene changed	
	to a <i>Sac</i> I site	
p6His-C	pATP19P containing a c-terminal 6x his-	(Jaschke and
	tagged RC-H gene as a 1.3 kb BamH I	Beatty 2007)
	fragment	- /

Plasmid	Relevant characteristics	Source or reference
pATSHR:: 6 his T ⁻ 3' sac	pATP19P containing the 1.3 kb BamH I –	This study
	Sac I fragment from pTZ18U::puhA T ⁻ 3'	
	sac	
pAli2::puf	pAli2 containing a 4.57 kb EcoR I	(Tehrani and
	fragment, encoding the R. sphaeroides	Beatty 2004)
	<i>pufQBALMX</i> genes	5 /
pAli2::puf sph ⁻	PAli2::puf with a 936 bp Sph I fragment	This study
	removed from the <i>pufQBALMX</i> insert	
pAli2::puf sph ⁻ (M)L214G	pAli2::puf sph ⁻ containing an L -> G	This study
	mutation at position 214 on the <i>pufM</i> gene	
pAli2::puf sph ⁻ (M)L214A	pAli2::puf sph ⁻ containing an L -> A	This study
	mutation at position 214 on the <i>pufM</i> gene	
pAli2::puf sph ⁻ (M)L214C	pAli2::puf sph ⁻ containing an L -> C	This study
	mutation at position 214 on the <i>pufM</i> gene	
pAli2::puf sph ⁻ (M)L214V	pAli2::puf sph ⁻ containing an L -> V	This study
	mutation at position 214 on the <i>pufM</i> gene	
pAli2::puf sph ⁻ (M)L214I	pAli2::puf sph ⁻ containing an L -> I	This study
	mutation at position 214 on the <i>pufM</i> gene	
pAli2::puf sph ⁻ (M)L214M	pAli2::puf sph ⁻ containing an L -> M	This study
	mutation at position 214 on the <i>pufM</i> gene	
pAli2::puf sph ⁻ (M)L214N	pAli2::puf sph ⁻ containing an L -> N	This study
	mutation at position 214 on the <i>pufM</i> gene	
pAli2::puf sph ⁻ (M)L214Q	pAli2::puf sph ⁻ containing an L -> Q	This study
	mutation at position 214 on the <i>pufM</i> gene	
pAli2::puf sph ⁻ (M)L214H	pAli2::puf sph ⁻ containing an L -> H	This study
	mutation at position 214 on the <i>pufM</i> gene	
pRS1	pATSHR:: 6 his T ⁻ 3' sac containing a 3.3	This study
	kb Sac I – EcoR I fragment from pAli2::puf	
	sph ⁻ containing the <i>pufQBALMX</i> genes	

Plasmid	Relevant characteristics	Source or reference
pRS1:: (M)L214G	pRS1 containing the an L -> G mutation at	This study
	position 214 on the <i>pufM</i> gene	
pRS1:: (M)L214A	pRS1 containing the an L -> A mutation at	This study
	position 214 on the <i>pufM</i> gene	
pRS1:: (M)L214C	pRS1 containing the an L -> C mutation at	This study
	position 214 on the <i>pufM</i> gene	
pRS1:: (M)L214V	pRS1 containing the an L -> V mutation at	This study
	position 214 on the <i>pufM</i> gene	
pRS1:: (M)L214I	pRS1 containing the an $L \rightarrow I$ mutation at	This study
	position 214 on the <i>pufM</i> gene	
pRS1:: (M)L214M	pRS1 containing the an L -> M mutation at	This study
	position 214 on the <i>pufM</i> gene	
pRS1:: (M)L214N	pRS1 containing the an L -> N mutation at	This study
	position 214 on the <i>pufM</i> gene	
pRS1:: (M)L214Q	pRS1 containing the an L -> Q mutation at	This study
	position 214 on the <i>pufM</i> gene	
pRS1:: (M)L214H	pRS1 containing the an L -> H mutation at	This study
	position 214 on the <i>pufM</i> gene	

2.2 Cloning. Plasmid DNA smaller than 10 kb was isolated using mini spin columns (Epoch Biolabs). For plasmids greater than 10 kb (e.g. pATP19P), the spin columns did not appear to work at all. Thus, these plasmids were purified using the alkaline lysis procedure outlined in Sambrook et al., however the final cesium chloride purification step was not performed (Sambrook et al. 1989). All restriction digests were performed using restriction enzymes purchased from NEB and used according to the manufacturer's instructions. T4 DNA ligase was also purchased from NEB and used according to the manufacturer's instructions with a few exceptions. Room temperature ligation reactions were performed for a full hour instead of 10 minutes. For overnight reactions, ligations were carried out in an ice bucket stored in a room maintained at 4 °C. Because cloning reactions involving large fragments of DNA (> 10 kb) were prone to much failure and troubleshooting, The following procedure was used to make such constructs:

- The vector and insert were combined into a single microcentrifuge tube at a molar ratio of ~1:5 vector to insert.
- 2. The combined vector and insert DNA were digested with restriction enzymes in a 20 μ L reaction.
- Following digestion, 2.5 μL of 10x ligase buffer, 1.5 μL of dH₂O, and 1 μL of T4 DNA ligase were added directly to the reaction tubes.
- 10 μL of the completed ligase reactions were used to transform CaCl₂ competent *E*. *coli*. It is highly recommended that the competency of the cells be higher than 10⁸ CFU/μg pUC19 plasmid DNA.

2.3 Modification of the *puhA* gene to facilitate the creation of RC expression

systems and purification of the RC. Nickel-NTA affinity chromatography of the R.

sphaeroides RC has been previously shown to be an effective tool in the rapid and relatively simple purification the protein compared to previous methods (Goldsmith and Boxer 1996).

Because the work presented in this thesis relies on the creation of many point mutations in the RC, measures were taken to ensure that the creation of RC expression systems was a streamlined process. The overall synthetic operon for RC expression is derived from a previous system used in this laboratory, and is illustrated in Figure 2.1.



Figure 2.1. General schematic for the design of a synthetic operon with the aim of expressing RCs in trans. The hypoxia-inducible *puc* promoter is denoted by the forward arrow, and the boxes represent the restriction fragments which include the *puhA* and *puf QBALMX* genes, respectively. Asterisks represent restriction sites for mobilization of genetic elements into the expression vector.

The first roadblock in creating this expression system was a putative rho-independent transcriptional terminator present immediately 3' of the *puhA* stop codon, between the codon itself and the natural *Bam*H I restriction site. Secondary structure predictions using the RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi, (Gruber et al. 2008)) of the 84 bp that constitute this region indicated the presence of a large hairpin structure present within the first 40 bp of this 3' region, shown in Figure 2.2 (Hofacker et al. 1994; Zuker and Stiegler 1981; McCaskill 1990). Transcription termination at this point would result in a large decrease or lack of *pufQBALMX* transcription, thus the terminator sequence needed to be removed.



Figure 2.2. Predicted secondary structure of the region 3' of the *puhA* stop codon. This stretch of sequence lies between the end of the coding sequence and the *Bam*H I site. The 5' and 3' ends of the sequence are denoted by the arrows. The large hairpin loop occurring within the first 40 bp is shaded in red.

The removal of this putative hairpin terminator was accomplished via site-directed mutagenesis. The so-called SLIM method (Chiu et al. 2004)for site-directed mutagenesis was employed for two reasons: (i) the amplification reaction did not rely on a relatively low frequency event in which the flanking sequence of the region to be deleted must bind to the primer throughout each amplification cycle, and (ii) the SLIM technique is capable of exponentially amplifying the mutagenic product, resulting in higher transformation efficiencies than other site-directed mutagenesis methods which employ linear amplification. This technique relies on the use of four primers: a forward and reverse "tailed" primer pair that incorporate the desired change in addition to ~20 bp of sequence complementary to the template, and two "short" primers which only contain the complementary sequence of the "tailed" primers. A schematic of this procedure is shown in Figure 2.3 A.



Figure 2.3. A schematic of the SLIM protocol for site-directed mutagenesis. (A) The forward and reverse tailed primer pair (F_T and R_T) incorporating the desired changes (the checkered portion of the primer) are used in a PCR reaction with short primers (F_S and R_S) that lack these regions. The reaction produces two productive and two non-productive PCR products which are *Dpn* I digested, and subsequently heated and cooled to yield self-annealing, transformable nicked plasmids. (B) Application of SLIM to produce a deletion of any size. The checkered region of the tailed primers is simply a stretch of sequence flanking the region to be deleted (dotted line).

The mutagenesis reaction, described in detail in Chiu et al. (Chiu et al. 2004), creates four amplification products. Two of the amplification products (those created from the binding of one "tailed" and one "short" primer to the DNA template) are linear copies of the DNA template containing the desired change on either end. After *Dpn* I digestion of the template DNA, these two products can be repeatedly heated and cooled to give products of DNA that are capable of self-annealing, yielding a nicked, transformable plasmid which contains the desired change. In the case of a deletion (Figure 2.3 B), flanking sequence from the region to be deleted is incorporated into the tailed primers. The result is a PCR reaction in which the region to be deleted is simply excluded from the amplification. Deletion of the hairpin sequence 3' of the *puhA* gene was accomplished with the primers listed in Table 2.3.

Primer name	Sequence (5' -> 3')
puhA T- SLIM F _T	GGATCCGATCCGCATCCGATCCGCCGCAGACGCACCCG
puhA T- SLIM Fs	ATCCGCCGCAGACGCACCCG
puhA T- SLIM R _T	CGGATGCGGATCGGATCCGATCAGGCGTATTCGGCCAG
puhA T- SLIM R _S	GATCAGGCGTATTCGGCCAG

 Table 2.3. Primers used for the deletion of the hairpin 3' of puhA

PCR amplification of the GC-rich genes from the *R. sphaeroides* requires the use of certain additives to the reaction mixture in order to ensure a successful reaction. For this reason, all mutagenic PCRs in this study were performed with the addition of 5% DMSO (final concentration), as well as 2 μ l of 100 mM MgSO₄ (NEB). Aside from these modifications, the mutagenic PCR reaction for the deletion of the hairpin region 3' of the *puhA* gene was performed under the same conditions described in Chiu et al (Chiu et al. 2004). Removal of this terminator sequence resulted in the creation of the plasmid pTZ18U::*puhA* T⁻.

Following the removal of the hairpin 3' of the *puhA* gene, a 6x his tag was added to the C-terminus of the *puhA* gene on pTZ18U::*puhA* T⁻ to allow for purification of the RC via metal affinity chromatography. The same mutagenic technique was employed again in order to add the histidine tag. This tag was added immediately 5' of the stop codon for the *puhA* gene. In addition to the tag, the penultimate GCC codon of the *puhA* gene was changed to GTG, creating an *ApaL* I restriction site (GTGCAC) that allowed for a simple means of screening for clones. The primers utilized for the addition of this tag are listed in Table 2.4. The PCR amplification of 6x his tagged *puhA* gene was performed under the same conditions as those employed for the removal of the hairpin terminator. Successful cloning of the mutagenic PCR product resulted in the creation of the plasmid pTZ18U::6his *puhA* T⁻.

Primer name	Sequence (5' -> 3')
6his PuhA T-	GTGCACCACCACCACCACTGATCGGATCCTCTAGAGTC
SLIM F _T	
6his PuhA T-	TGATCGGATCCTCTAGAGTC
SLIM F _S	
6his PuhA T-	GTGGTGGTGGTGGTGGTGCACGTATTCGGCCAGCATCGC
SLIM R _T	
6his PuhA T-	GTATTCGGCCAGCATCGC
SLIM R _s	

Table 2.4. Primers utilized for the addition of a C-terminal 6x his tag

Attempts to clone the *puhA* gene from pTZ18U::6 his *puhA* T⁻ as a *Bam*H I fragment into plasmid pATP19P repeatedly resulted in either a complete cloning failure or a preference for the fragment to insert in the wrong direction. For this reason, the *Bam*HI site downstream of the gene was changed to a *Sac*I site using the SLIM method once more. The primers used for this reaction are listed in Table 2.5. The PCR reaction conditions are the same as above. A successful mutagenesis reaction resulted in the creation of the plasmid pTZ18U::6 his *puhA* T⁻ 3' Sac. This plasmid allowed for the insertion of the *puhA* gene into pATP19P as a *Bam*H I to *Sac*I fragment, generating the plasmid pATSHR:: 6 his T⁻ 3' sac.

Primer	Sequence (5' -> 3')
name	
Bam2Sac	CACTGATCGAGCTCTCTAGAGTCGACCTGCAGGCATGCAAGCT
PuhA F _T	
Bam2Sac	TCGACCTGCAGGCATGCAAGCT
PuhA Fs	
Bam2Sac	CTCTAGAGAGCTCGATCAGTGGTGGTGGTGGTGGTGCACGTATTC
PuhA R _T	
Bam2Sac	GTGGTGGTGGTGGTGCACGTATTC
PuhA R _s	

Table 2.5 Primers utilized to change the *Bam*H I site 3' of the *puhA* gene to a *Sac* I site

2.4 Removal of excess DNA from pAli2::puf. The plasmid pAli2::puf contained the *pufQBALMX* operon present as a large 4.57 kb *Eco*R I fragment. Because this plasmid was going to be utilized for PCR based mutagenesis, it was helpful to remove some of the excess DNA, which would facilitate any PCR-based methods applied on the plasmid. While there did not seem to be any obvious methods of DNA removal with restriction enzymes for the region upstream of the *pufQBALMX* operon, the sequence downstream of the operon contained 936 bp of sequence flanked by *Sph* I sites. Thus, pAli2::puf was simply digested with *Sph* I (NEB) and re-ligated with T4 DNA ligase (NEB), generating the plasmid pAli2::puf sph⁻.

2.5 Mutagenesis of the reaction center M gene. In order to change the residue at position 214 on the M subunit of the RC from *R. sphaeroides*, the plasmid pAli2::puf sph⁻, which contains the *pufQBALMX* operon was utilized for site-directed mutagenesis. Because this series of mutations consisted of single codon changes, the more simple Quickchange protocol from Stratagene was used. In this method, two complementary oligonucleotides containing the point mutation of interest at the center are utilized such that they can bind a DNA template through their flanking sequence. Amplification of a template with such primers results in the production of single-stranded products which are capable of self-annealing, generating a nicked plasmid product. Since the amplification reaction is linear (i.e. the primers can only generate useful product when bound to the original starting template), more starting material is required for the reaction. In addition, fewer amplification cycles are employed since it has previously been shown that the proofreading polymerase that I used for these mutagenic PCR reactions (Vent from NEB) begins to degrade the amplified product when 20 or more cycles are used in the PCR reaction (Byrappa et al. 1995). Mutagenic PCR

reactions for point mutations in the RC M gene were set up as follows: 50-100 ng of starting template DNA, 1 μ L of each mutagenic primer (10 μ M stock concentration), 1 μ L of dNTPs (5 mM stock), 5 μ L of 10x Thermopol buffer (NEB), 2.5 μ L of DMSO (Acros Organics), 2 μ L of 100 mM MgSO₄ (NEB), 1 unit of Vent polymerase (NEB), and dH₂O to 50 μ L. A table of the primers used in this study to change the (M)L214 residue to different amino acids is listed in Table 2.6.

Table 2.6. Primers used to change the (I	M)L214 residue on the RC M gene.
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Primer	Desired	Forward primer *
name	residue	
(M)L214G	Glycine	5' CTCTACGGGTCGGCCGGCCTCTTCGCGATGCAC 3'
(M)L214A	Alanine	5' CTCTACGGGTCGGCCGCCCCTCTTCGCGATGCAC 3'
(M)L214C	Cysteine	5' CTCTACGGGTCGGCC TGC CTCTTCGCGATGCAC 3'
(M)L214V	Valine	5' CTCTACGGGTCGGCCGTCCTCTTCGCGATGCAC 3'
(M)L214I	Isoleucine	5' CTCTACGGGTCGGCCATCCTCTTCGCGATGCAC 3'
(M)L214M	Methionine	5' CTCTACGGGTCGGCCATGCTCTTCGCGATGCAC 3'
(M)L214N	Asparagine	5' CTCTACGGGTCGGCCAACCTCTTCGCGATGCAC 3'
(M)L214Q	Glutamine	5' CTCTACGGGTCGGCCCAGCTCTTCGCGATGCAC 3'
(M)L214H	Histidine	5' CTCTACGGGTCGGCCCACCTCTTCGCGATGCAC 3'

* Reverse primers were the reverse complement of the forward primer
The reaction conditions for the (M)L214 mutagenic PCR reactions proceeded as follows. An initial denaturation step was performed at 95 °C for 3 minutes. This was followed by 15 cycles of: 95 °C for 20 seconds, 50 °C for 20 seconds, and 68 °C for 5 minutes and 50 seconds. After the amplification step, the reaction was held at 68 °C for 7 minutes and then held at 10 °C indefinitely. Following the PCR, 0.5 μ L of *Dpn* I enzyme (NEB) was added to each reaction mixture without any further purification in order to remove any remaining template DNA. Due to the increased amount of template required for the reactions, the restriction digests were incubated for two hours or overnight in order to ensure complete digestion of the template DNA. Ten microliters of each of these restriction digests were subsequently used to transform CaCl₂ competent *E. coli*.

2.6. Assembly of the RC genes onto a broad host range expression vector for *in trans* expression of mutant RCs. The cloning of the *pufQBALMX* genes into a broad host range vector was previously achieved via mobilization of the operon from pAli2::puf as an *Eco*R I fragment. This method, however, failed to succeed in my hands because of a tendency for this fragment to always insert in the wrong orientation. Because of this, the *pufQBALMX* genes on pAli2::puf sph⁻ (M)L214X (where X denotes the single letter notation of the amino acid replacing leucine at position 214 on the RC M gene) were subcloned into pATSHR:: 6 his T⁻ 3' sac as *Sac* I to *Eco*R I fragments. The cloning method described in section 2.3 was used, as it gave good success in cloning the *puhA* gene into pATP19P. The resultant plasmid was called pRS1::(M)L214X. A map of this plasmid is shown in Figure 2.4.



Figure 2.4. A genetic and restriction map of pRS1. The bent arrow indicates the *puc* operon promoter. Curved arrows represent transcribed genes from the plasmid. Tc = tetracycline resistance.

2.7. Purification of the RC. Reaction centers were purified using a modified version of the protocol of Goldsmith and Boxer (Goldsmith and Boxer 1996). The cells from 21 L of R. sphaeroides semi-aerobic culture were collected by centrifugation and the cell paste was resuspended in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM MgCl₂ to a volume of 175 mL. For the purification of Zn-BChl-containing RCs, the culture volume was up-scaled to 72 liters in order to obtain amounts of protein for spectroscopic analyses. A few crystals of DNase I were added to the suspension before lysing the cells in a French press at 18,000 PSI. Lysed cells were centrifuged to pellet debris and unbroken cells. The supernatant was then centrifuged at $66,226 \times g$ at $4^{\circ}C$ overnight to pellet chromatophores. Following ultracentrifugation, the supernatant was discarded and the remaining pellet was resuspended in 10 mM Tris, pH 8.0, 150 mM NaCl, to a volume of 100 mL. Samples (1 mL) of this suspension were placed in 1.7 mL microcentrifuge tubes, brought to room temperature, and lauryldimethylamine oxide (LDAO; Fluka) was added to each tube at concentrations ranging from 0.5% to 2.75% to determine the optimal concentration of detergent for RC solubilization. These samples were rocked in the dark for 30 minutes, and 900 μ L were centrifuged at $107,400 \times g$ for 30 min at 4°C. The 875 nm absorbance maximum of the supernatants was used as a measure of the degree of photosynthetic complex solubilization. In our experiments, the optimal concentration of LDAO for chromatophore solubilization varied between 1.25% and 1.75% LDAO. Once the optimal LDAO concentration was found, the remaining chromatophore suspension was brought to this detergent concentration and stirred in the dark for 30 min at room temperature, and centrifuged at $117,734 \times g$ at 4 °C for 15 min. The supernatant solution was collected and imidazole (10 mM) and NaCl (200 mM) added to the concentrations indicated before loading onto a Ni NTA column (Qiagen). The

column was washed with 10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% LDAO until the maximum absorbance over the range of 500 - 950 nm was less than 0.01. The RC was eluted from this column with 10 mM Tris, pH 8.0, 150 mM NaCl, 300 mM imidazole, 0.1% LDAO, and dialyzed against TL buffer (10 mM Tris, pH 8.0, 0.1% LDAO). For crystallization, RCs were purified further by anion exchange chromatography with an ÄKTA Explorer FPLC system (GE Healthcare) equipped with a SourceQ column and elution with a NaCl gradient (0 – 200 mM), with the majority of RCs eluting at 120 mM NaCl. The eluted RCs were desalted by passage through a 10DG column (Bio Rad) and concentrated with an Amicon 30 kDa centrifugal ultrafilter (Millipore). RCs used for crystallization experiments were not frozen and used immediately following purification.

2.8. Quantification of pigments from RCs. Pigments of RCs were extracted with a solution of acetone:methanol (7:2) and quantified according to Van der Rest and Gingras (Van der Rest and Gingras 1974). Following the extraction, the samples were briefly centrifuged to remove a precipitate that formed upon addition of the organic solvent to the RC samples. Absorbance spectra of the isolated pigments were obtained on a Hitachi U-3010 spectrophotometer (Hitachi).

2.9. Low temperature absorption spectroscopy. Cryogenic absorbance spectra (0.6 nm bandwidth) were obtained at ~10-13 K using a Cary 6000 spectrophotometer (Agilent) equipped with a closed-cycle helium cryostat (Omniplex OM-8; ARS Inc.) as described by Lin *et al.* (Lin et al. 2009). RC samples in 10 mM Tris buffer, pH 8.0, 0.1% LDAO were concentrated to an A_{802} of ~40 by centrifugation using a 30 kDa cut-off Centricon ultrafilter (Millipore). Samples were supplemented with sodium ascorbate (final concentration of 1

mM) and diluted 1:1 with spectroscopic grade glycerol prior to freezing between two quartz plates separated by a \sim 25 µm polycarbonate spacer.

2.10. X-ray crystallography. Fresh solutions of RC purified as described above were used to set up hanging drop crystallization trials. Instead of mixing a reservoir solution with the protein, a separate precipitant solution (1 M potassium phosphate pH 7.4, 3.5% 1,2,3heptanetriol, and 0.1% LDAO) was prepared and mixed with the protein solution on cover slips before sealing over the reservoir. Various ratios of precipitant and protein solution were found to result in crystals of widely varying quality. Diffraction quality crystals of (M)L214G ($\sim 0.3 \times 0.3 \times 0.3$ mm) grew at a ratio of 2 µL RC solution (7 mg/mL, or 20.6 OD₈₀₂/mL):1 µL precipitant solution at 298 K, protected from light, over a reservoir of 1.52 M potassium phosphate buffer, pH 7.4. Crystals appeared after approximately 72 hours. Repeated exposure of the crystals to light appeared to diminish diffraction quality, so immediately after initial exposure to light, crystals were transferred to a solution of 1 M potassium phosphate buffer (pH 7.4) and 30% glycerol before flash-freezing in liquid nitrogen. The (M)L214A and (M)L214N crystals were produced and frozen in a similar fashion, except that the former grew from a 5 mg/mL protein solution at a 3:1 protein:precipitant ratio, whereas the (M)L214N crystals formed from a 3 mg/mL protein solution at a 2:1 drop ratio, over a reservoir of 1.5 M potassium phosphate buffer, pH 7.4.

Diffraction data from a single (M)L214G crystal were collected at the Canadian Light Source on beamline ID-1 and processed using AUTOXDS, Pointless, and Scala to 2.2 Å resolution (Kabsch 2010; Winn et al. 2011). Data sets for (M)L214A and (M)L214N crystals were collected at the Stanford Synchrotron Radiation Lightsource on beamline 7-1 and processed using HKL2000 (Otwinowski and Minor 1997) for (M)L214A, or using Mosflm,

Pointless and Scala for (M)L214N (Winn et al. 2011; Leslie and Powell 2007). All three mutant RCs crystallized in the space group $P3_121$, with one molecule in the asymmetric unit. Solvent content was approximately 75%, which is typical for a membrane protein crystal. All data sets were isomorphous with the wild type (WT) RC (PDB entry 2J8C, chosen for comparison because it is the highest resolution (1.85 Å) RC structure in the PDB). The (M)214 mutant structures initially underwent five cycles of rigid body refinement with Refmac5 (Murshudov et al. 1997); the input model used was an edited version of PDB entry 2J8C, with residue (M)L214 changed to the appropriate side chain. Subsequently, models were improved first by alternating between inspection of $2F_0$ - F_c and F_0 - F_c electron density maps and manual editing in Coot (Emsley and Cowtan 2004), and followed by restrained refinement with Refmac5. Manual editing consisted primarily of adding and removing solvent molecules (mainly water, but also glycerol, 1,2,3-heptanetriol, and LDAO). Water molecules were added using peaks $> 3\sigma$ in the F_0 - F_c difference map with plausible hydrogen bonding geometry. Omit difference density maps, in which the phytyl tail of B_A and the nearby LDAO molecule were omitted from the refinement model, were generated to remove model bias from the refinement procedure. To produce these maps, the occupancies of the phytyl tail atoms from CGA to C20, as well as LDAO and/or glycerol, were set to 0, and the model was refined by 8 cycles of restrained refinement in Refmac5. Unweighted omit maps were generated using the FFT program in the CCP4 suite (Winn et al. 2011).

For the Zn-BChl-containing RCs, diffraction data were collected at Stanford Synchrotron Light Source on beamline 7.1 using a wavelength of 1.12709 Å and processed with Mosflm and Aimless to 2.85 Å resolution (Leslie and Powell 2007; Winn et al. 2011). Anomalous differences were detected to 6 Å resolution. All data sets were isomorphous with wild type RC (PDB entry 2J8C), which was used as the starting point for refinement with removal of the Mg²⁺ ions and for Zn- β -RC the side chain atoms of (M)214. Initial phases were obtained by limited refinement with Refmac5 (Vagin et al. 2004) and F_0 - F_c and anomalous difference maps were computed with Coot (Emsley and Cowtan 2004) and FFT, respectively. Based on the peaks observed in both maps, Zn-BChl was modeled at each of the P, B, and H cofactor sites in both the A and B branches. Density for a histidine side chain was observed at residue (M)214 in the Zn- β -RC and modeled accordingly using the program Coot. Refinement of the structures was continued with Refmac5 and the removal of solvent atoms with unrealistic B-factors.

2.11. Photoheterotrophic growth experiments. For photoheterotrophic growth of *R*. *sphaeroides*, inoculum cultures were grown in RCV medium aerobically on a rotary shaker at 200 RPM. Two hundred μ L of this culture were used to seed 16.5 mL photoheterotrophic starter cultures in screw cap tubes at 30 °C. These tubes were placed under saturating light intensity for 48 hours to adapt cells to photoheterotrophic conditions prior to low light growth. Adapted cultures were transferred to fresh 16.5 mL screw cap tubes and diluted with RCV medium to a density of approximately 30 Klett units. Cultures were grown at a light intensity of 5 μ Em⁻²s⁻¹ (measured with a LI-185B photometer; Li-Cor) and culture density measurements were taken with a Klett photoelectric colorimeter (100 Klett units = ~3 X 10⁸ CFU/mL).

2.12 Time-resolved spectroscopy. Transient absorption measurements were performed at room temperature using a home-built spectrometer based on a kilohertz Ti:sapphire laser, according to the methods of Pan et al. (Pan et al. 2011; Pan et al. 2013). Briefly, Pulses of 1 mJ, at a repetition rate of 1 kHz (150 fs pulse duration at 800 nm), were generated from a

regenerative amplifier system (Tsunami and Spitfire, Spectra-Physics). Part of this pulse energy was used to pump an optical parametric amplifier to generate 865 nm excitation pulses. The broadband probe pulse was generated by focusing a weak 800-nm beam into a 3mm sapphire plate, and sent to an optical compressor composed of a pair of prisms, before it was focused onto the sample. The white-light probe pulses were then dispersed by a spectrograph and detected using a charge-coupled device camera (DU420, Andor Technology). The polarization of the pump pulse was set to the magic angle (54.7°) with respect to the probe pulse. Samples were loaded onto a spinning wheel with a path length of of 1.2 mm. Global analysis of the time-resolved spectra was performed using ASUFIT, a MATLAB program provided by Dr. Su Lin at Arizona State University. The quinone-lacking RC control for transient spectroscopy measurements was prepared by treatment of native RCs with 4% LDAO and 10 mM o-phenanthroline (Sigma) for three hours in the dark, according to the methods of Okamura et al (Okamura et al. 1975).

2.13. Partial purification of chlorins. A novel method of partial pigment purification was devised for the extraction of BChl *a* from photosynthetic bacteria. Existing methods rely on the use of large amounts of acetone:methanol mixtures, followed by a tedious drying step which is conducive to chlorin allomerization from exposure to light and oxygen (Gauthier-Jaques et al. 2001), or via a lengthy precipitation procedure involving the carcinogenic chemical dioxane, which also involves extended periods of time in which the chlorins are exposed to air and light (Iriyama et al. 1974). In this new protocol, a combination of solvent and detergent removes the chlorin from the broken cell mixture, yielding a high concentration of pigments in a small volume. The solvent extraction and drying steps are

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very short, and do not allow for much chlorin allomerization, even when working under aerobic conditions in the light.

BChl a was purified from photosynthetically grown R. capsulatus strain $\Delta gtaI \Delta 1081$, because this strain achieves unusually high cell densities, thus yielding more BChl per liter of culture compared to wild type R. capsulatus or R. sphaeroides cells. In addition, this strain does not suffer from centrifugation issues stemming from capsular polysaccharide production (Brimacombe et al. 2013). Cells were centrifuged at 7000 x g in 1 L bottles, in a JLA 8.1 rotor (Beckman Coulter). The supernatant was discarded, and cells were resuspended in ~ 30 mL of 10 mM Tris, pH 8.0, 150 mM NaCl, 2 mM MgCl₂. A few crystals of lyophilized DNAse was added to the cell suspension before lysis via a single passage through a French press. Cell debris was removed by discarding the pellet after centrifugation at 10,000 x g for 10 minutes in a JA-20 rotor (Beckman Coulter). To the remaining supernatant, LDAO was added to a final concentration of 1.5-2%, and 1/5th to 1/10th volume of ether was added to the mixture. The resultant mixture was poured into a 50 mL screw cap graduated polypropylene tube, inverted several times, and allowed to settle. The upper ether layer containing the pigments was removed, dried under nitrogen and resuspended in a desired volume of methanol.

For the partial purification of Chl *a*, the detergent/ether method was not tested, and thus an acetone:methanol extraction method was used. 100 g of dandelion leaves were harvested from plants growing rampant around campus. The leaves were cut into small pieces with a pair of scissors, placed in a 1 L beaker, and dried as much as possible with paper towels. It is important to note that water was not completely removed in this step, and the remaining water assisted in the precipitation of the pigments. To the partially dried leaf

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pieces, 500 mL of ice cold 7:2 acetone:methanol was added. The plant cell suspension was then sonicated to assist in cell rupturing. The mixture was stored at -20 °C in the dark for one hour. Leaf pieces were removed from the solvent by passing the suspension through a piece of filter paper placed inside a glass powder funnel. The filtrate was then dried under a stream of nitrogen until the pigments precipitated out of solution from the gradually increasing water concentration in the drying mixture. These pigments were rapidly collected by laying a piece of filter paper on a porcelain Buchner funnel and slowly pouring the pigment solution onto the paper. The filter paper was placed in a Petri dish, and ~ 10 mL of acetone was added to the dish, removing the majority of the pigments from the filter paper. The pigments in acetone were transferred to a glass test tube, dried under nitrogen, and resuspended in methanol.

2.14. Semi-preparative HPLC purification and quantification of chlorins. For the purification of Chl *a* and BChl *a*, 1.5 mL of partially purified pigments in methanol were filtered through a 0.45 μ m nylon membrane. The filtrate was placed in a glass HPLC vial, and injected into a Waters 2695 HPLC equipped with a photodiode array (Waters) and a semi-preparative C18 column (Luna C18, 250 x 10, 5 μ m bead size), using 100% methanol as the mobile phase (Shioi et al. 1983). For the purification Chl *a*, the detection wavelength was set to 666 nm. For BChl *a*, the detection wavelength was set to 771 nm. Concentrations of collected Chls and BChls were determined by absorption spectroscopy using their extinction coefficients in methanol (79.95 and 60 1 g⁻¹ cm⁻¹ for Chl *a* and BChl *a*, respectively (Namsaraev 2009)). The concentration was calculated as C = A/a, where C is the concentration of the solute in mg/sample, A is the absorbance value at 665 or 771 nm for Chl *a* and BChl *a*, respectively, and a is the extinction coefficient.

2.15. Pheophytinization of chlorins by chemical means. Chl *a* and BChl *a* were pheophytinized by the addition of 3 μ L of concentrated HCl per 100 μ L of pigment solution. This mixture was a allowed to sit for 5 minutes on ice in the dark before the solvent was completely dried under a stream of nitrogen gas. The dried pigments were resuspended in the desired volume of solvent.

2.16. Pigment substitution of RCs under mild conditions. The protocol for the substitution of pigments was modified from the methods of Franken et al. since the (M)L214G mutant RC denatured under these conditions (Franken et al. 1997). Purified pigments were concentrated by drying and subsequent resuspension in acetone, such that 50 μ L of this concentrate contained a 20-fold molar excess of pigments compared to RC. The pigment concentrate was combined with a buffer consisting of 10 mM Tris, pH 8.0, 0.1% LDAO. To this mixture, 0.5 mg of RC was added. The final volume of the reaction was 500 ul. Once set up, the reactions were stored in the dark at room temperature for 2 hours. Following this incubation, the reactions were transferred into multiple PCR tubes and incubated at 37 °C in a PCR machine for 90 minutes. To remove excess pigments and denatured protein, the reactions were purified by anion exchange chromatography using DEAE 650M (Tosoh). The resin was equilibrated in 10 mM Tris, pH 8.0, 0.1% LDAO, after which the reaction mixtures were bound to the column. RCs bound to the column were washed with 10 column volumes of 10 mM Tris, pH 8.0, 0.1% LDAO, 25 mM NaCl. The protein was eluted with the same buffer containing 120 mM NaCl.

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3. RESULTS

3.1 Optimization of chemotrophic growth conditions for *R. sphaeroides*. Despite having a functional expression system for the purification of the RC, initial attempts at purifying the pigment-protein complex produced yields that were not sufficient to provide enough starting material for the spectroscopic and crystallographic analyses used in this study. The existing protocol used in the laboratory for the chemotrophic (semi-aerobic in the dark) growth of *R. sphaeroides* cultures employed 2 L Erlenmeyer flasks filled to 80% nominal capacity with LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl), shaken at 150 RPM. This technique produced cultures that were approximately 220 – 300 Klett units in cell density after 96 hours. In this study, the growth conditions were optimized by changing the culture medium and volume such that higher cell densities would be attained upon harvesting.

Optimization of the culture growth medium began with the observation that R. sphaeroides cultures appeared to contain stronger LH1 absorbance intensity (measured at 875 nm) when grown in the synthetic medium RCV than in the complex medium LB, after 96 hours. Because the production of > 20 L of a synthetic medium like RCV is both labor intensive and costly, I wanted to see if one or more of the ingredients of RCV medium was responsible for the observed increase in LH1 peak intensity per mL of cell culture. Thus, flasks were prepared in which selected RCV components were added to LB medium (in the same concentration as in RCV medium). The cultures were allowed to grow for 96 hours before a whole cell absorption spectrum was taken. In addition, a sample was included in which the culture was grown in LB medium in complete darkness by wrapping the entire flask in aluminum foil. Because light negatively regulates RC-LH1 complex production, this sample was included to test whether greater quantities of RCs can be expressed from samples grown in complete darkness (Shimada et al. 1992). The results are compiled in Figure 3.1.



Figure 3.1. Whole cell absorption spectra of chemotrophically grown *R. sphaeroides* cultures incubated in the presence of various RCV media components. The spectra were normalized to $A_{650} = 0.2$. LB = LB medium. RCV = RCV medium. LB Dark = LB culture grown in complete darkness. Each variable culture had one component of RCV added to LB in its respective final concentration in the synthetic medium (See Section 2.1 for the exact concentrations of each solute). Super salts contains: MgSO₄, CaCl₂, FeSO₄, EDTA, trace elements, and thiamine hydrochloride. Absorption spectra were taken after 96 hours of growth.

The results in Figure 3.1 show that one of the RCV medium components, Super Salts, had a relatively large stimulatory effect on the LH1 peak. Because the amplitude of this peak is proportional to the amount of the RCs in a sample, a larger LH1 peak intensity is indicative of a greater amount of the RC. Also, because Super Salts consists of a mixture of magnesium, calcium, iron, thiamine, and several trace elements, a subsequent experiment of the same fashion was performed in which each of the Super Salts components was added separately to individual flasks of LB medium. The results of this experiment are compiled in Figure 3.2.



Figure 3.2. Whole cell absorption spectra of chemotrophically grown *R. sphaeroides* cultures grown in the presence of various Super Salts components. The spectra were normalized to $A_{650} = 0.2$. Each variable culture had one component of Super Salts added to LB in its respective final concentration in the synthetic medium RCV (See Section 2.1 for the exact concentrations of each solute). Absorption spectra were taken after 96 hours of growth.

The results in Figure 3.2 show that three constituents of the Super Salts mixture had stimulatory effects on *R. sphaeroides* LH1 peak intensity after 96 hours of growth: magnesium, calcium, and thiamine. Although this growth enhancement was reproducible with magnesium and calcium, the thiamine effect was not reproducible. Thus, thiamine was ruled out as having a stimulatory effect on chemotrophic LH1 production in *R. sphaeroides* cultures, and only magnesium and calcium were used as enhancers in the growth medium of preparative-scale *R. sphaeroides* cultures. As a final modification to the medium recipe, the amount of sodium chloride was reduced from ~170 mM to 4 mM because it was discovered over half a century ago that *R. sphaeroides* grows optimally at much lower salt concentrations (Sistrom 1960). The final composition of the so-called RLB medium consists of: 10% tryptone, 5% yeast extract, 4 mM NaCl, 810 µM MgCl₂, and 510 µM CaCl₂.(Jun et al. 2013)

Following the development of a better medium for the growth of *R. sphaeroides*, I sought to investigate whether a greater amount of the RC could be produced from RLB cultures when the volume of medium in the flask was changed. The rationale behind this idea was that chemotrophic growth of *R. sphaeroides* cultures in flasks filled to 80% nominal capacity did not produce culture densities as high as the same cultures grown aerobically or photoheterotrophically (anaerobic under illumination). Because filling an Erlenmeyer flask beyond 80% nominal capacity would result in little to no aeration at 150 rpm, the flask volume was titrated from 50% to 80% nominal capacity. After 48 hours of growth, the cells in all the flasks began to turn red, becoming a deeper red after 72 hours of growth. Therefore, measurements were taken after 72 hours of growth, with a subsequent measurement taken after 96 hours. The results are displayed in terms of a factor called "LH1 output". This term

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takes into account culture density as well as LH1 absorbance amplitude at 875 nm to compare the relative amounts of LH1 in a given volume of culture. The absorbance of LH1 at 875 nm was used as an estimate for RC concentrations because of the stoichiometric relationship between LH1 and the RC in core complexes of *R. sphaeroides*. Because cell light-scattering also contributes to the absorbance value at 875 nm, this scattering component was subtracted. This was done by estimating an 875 nm scatter value, which was calculated as the mean of the absorbance at 825 nm and 925 nm (assuming that the amount of lightscattering is linear along this portion of the near IR spectrum), and subtracting the 875 nm scatter value from the 875 nm absorbance to yield an estimated 875 nm absorbance value attributed to LH1 light absorbance alone. This LH1 contribution was multiplied by the absorbance of the sample at 700 nm (a region of the spectrum where absorbance is affected by light scattering, and thus cell density, alone), yielding the "LH1 output" value. This was done in order to account for increases in RC production due to the presence of more cells per ml of culture. The equation for LH1 output is as follows:

LH1 output =
$$A_{700} * (A_{875} - ((A_{825} + A_{925}) / 2))$$
 [1]

The results of applying equation 1 to *R. sphaeroides* cultures grown in flasks filled with RLB media at 50% – 80% nominal capacity are presented in Figure 3.3.



Figure 3.3. Comparison of LH1 output values from cells grown in flasks filled to different volumes. The flasks were filled to 50%, 60%, 70%, or 80% nominal capacity with RLB medium. The first four bars represent samples taken at 72 h after inoculation, and the last four bars represent measurements after 96 h. Bars are averages of three independent measurements. Error bars represent standard deviations about the mean.

The results in Figure 3.3 demonstrate that much higher levels of LH1, and therefore RC, are achievable from cultures grown in RLB medium when a particular volume is used. Specifically, the flasks filled to 50%, 60%, and 70% nominal capacity produced two to three times as much LH1 per unit culture volume as the flasks filled to 80% nominal capacity. Between the 50%, 60%, and 70% samples, however, there did not seem to be a significant difference. Because of this, I chose to grow all future preparative scale *R. sphaeroides* cultures in Erlenmeyer flasks filled to 70% nominal capacity. This allowed me to use the most amount of media per flask, which in turn allowed me to grow the highest amount of cells in the limited shaker space provided in a shaker system that is shared among several laboratories.

The aim of the work presented above was not directly geared towards the answering of my research question. However, it enabled me to proceed faster through the subsequent experiments presented in Section 3 of this thesis. In addition, I have devised a simple and inexpensive means to grow preparative scale cultures of *R. sphaeroides*. Despite the fact that these growth conditions were only analyzed in this strain, I believe they have the potential to be used for other strains of purple bacteria, such as *R. capsulatus* and others.

3.2. Pigment composition of RCs with amino acid changes at position 214 on the

M subunit. Absorption spectroscopy of RC pigment extractions provides a simple and remarkably accurate tool to analyze the pigment content of RCs using the extinction coefficients of the isolated pigments in a 7:2 acetone:methanol mixture (Van der Rest and Gingras 1974). I applied this method in my analysis of RCs in which residue 214 of the M protein had been changed from Leu, designated as (M)214 mutants. The BChl:BPhe ratios of all (M)L214 mutant RCs are listed in Table 3.1. These data indicate that RCs containing

aliphatic or sulfur-containing side chains at (M)214 have a chlorin pigment composition that is the same as or close to that of the wild type RC (*i.e.*, 2 molecules of BChl for every molecule of BPhe). Conversely, mutants with amide-containing side chains at H_A (Asn, Gln) have a pigment composition that resembles that of the previously reported (M)L214H mutant (5 molecules of BChl for every molecule of BPhe (Kirmaier et al. 1991)).

Strain	BChl:BPhe Ratio*
wild type	2.03 ± 0.09
(M)L214G	2.60 ± 0.11
(M)L214A	2.20 ± 0.11
(M)L214C	2.19 ± 0.09
(M)L214V	2.19 ± 0.02
(M)L214I	2.15 ± 0.11
(M)L214M	2.39 ± 0.01
(M)L214H	4.74 ± 0.04
(M)L214N	4.70 ± 0.29
(M)L214Q	4.77 ± 0.08

Table 3.1. Pigment composition of (M)214 mutant RCs.

* Values are averages of three extractions \pm the standard deviation about the mean.

3.3 Ground-state analysis of mutant RCs by low temperature absorbance

spectroscopy. In order to gain more insight into the cofactor environments of the (M)L214 mutants, and to confirm the results of RC pigment extractions, absorption measurements of samples cooled using a helium cryostat were employed. Such techniques restrict protein dynamic heterogeneity, yielding high resolution spectra in which some, although not all, individual pigments in the RC are revealed as absorption peaks.

3.3.1 Effect of replacement of (M)L214 with nonpolar or S-containing amino acid residues on the Q_y and Q_x transitions of RC BPhes. The 11 K electronic absorbance spectra of RC mutants bearing nonpolar residues at (M)214 are compared in Figure 3.4. At this temperature, spectroscopic differences that were subtle in room temperature absorbance spectra became more evident. Nevertheless at ~760 nm, for example, the Qy transitions associated with the two BPhes in the wild type RC overlapped considerably, although absorbance on the red and blue edges of the peak corresponds to H_A and H_B, respectively (Figure 3.4A (Watson et al. 2005b)). All of the nonpolar (M)214 substitutions created in this work resulted in changes in the BPhe Qy absorbance region, ranging from almost imperceptible (in the (M)L214A RC) to most obvious in the (M)L214G/I/M RCs. Replacement of (M)L214 with Gly resulted in a 5 nm blue-shift of the BPhe Qy absorbance peak, from 759 to 754 nm, evidently due to a change in the H_A band. In the spectra of the (M)L214M and (M)L214A mutants the corresponding peak shifts were 3.9 nm and 0.4 nm, respectively; the overlap of the H_A and $H_B Q_y$ transitions was enhanced as a result, and the combined absorbance bands appeared more symmetrical. In contrast, the absorbance spectra of the RC mutants with Cys, Val or Ile in place of (M)L214 exhibited red-shifts of the H_A

BPhe Q_y transition maximum, thereby accentuating the difference between the Q_y absorbances of H_A and H_B .

Together, the above observations reflect primarily environmental perturbations of the H_A chlorin when (M)L214 is replaced with another nonpolar residue, which affect the wavelength of light that is absorbed. Unlike the changes observed in the spectrum of the (M)L214H mutant (see below), the magnitudes of these shifts are much smaller, indicating that the pigment at this site in each mutant RC was a BPhe rather than a BChl.

As shown in Figure 3.4B, the low temperature Q_x transitions of the wild type RC yielded three major absorbance peaks, and a long-wavelength shoulder. The peak near 535 nm is due to the H_B BPhe and the ~545 nm peak corresponds to the absorbance maximum of the H_A BPhe, whereas the peak and shoulder near 600 nm are due to BChls (Shochat et al. 1994; Kirmaier et al. 1985).

The (M)L214G and (M)L214A RCs exhibited a blue-shifting of the H_A BPhe peak, such that the two Q_x transitions merged to form a peak with a shoulder (Figure 3.4B). In contrast, the BChl region around 600 nm was not greatly affected. These data confirm our interpretation of the Q_y data: although the electronic properties of H_A are affected when (M)L214 is substituted by small, nonpolar moieties such as Gly and Ala, the chlorin in the H_A site remains a BPhe. The H_B and H_A Q_x transitions of the (M)L214C/V/I/M mutants were relatively similar to each other and to the wild type RC, in peak amplitude and wavelength, indicating that the H_A site of these mutants contains BPhe.

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Figure 3.4. Low temperature (~11 K) ground-state absorption spectra of (A) the Qy and (B) the Qx transitions of the nonpolar (M)214 mutant series. Spectra were normalized to the Q_y absorption maximum of the dimer (P) BChl peak at ~895 nm. Peak assignments for the spectra can be found in Figure 1.8. The dashed lines mark the Q_x and Q_y absorption peak wavelengths of H_A as well as the peak wavelengths for the Q_y transition of P and B_A/B_B, in the spectrum of wild type RC. Reprinted with permission from Saer et al. Copyright 2013 American Chemical Society (Saer et al. 2013a).

3.3.2 Effect of replacement of (M)L214 with nonpolar or S-containing amino acid residues on the Q_y and Q_x transitions of RC BChls. Because the (M)214 side chain is relatively distant from the RC BChl macrocycles, it was surprising to discover striking differences between the absorbance of wild type and some nonpolar RCs in the $\sim 802 \text{ nm } O_v$ transition region of the B_A/B_B accessory BChls. In the low temperature absorbance spectrum of the wild type RC, there is a major peak (attributed to a combination of B_A and B_B) that has a distinct shoulder on the long wavelength side, which has been attributed to B_B, the BChl in the inactive B branch (Kirmaier et al. 1985; Neupane et al. 2012). It is interesting to note that the magnitude of this shoulder varies between low temperature ground state absorbance spectra of the wild type RC obtained by different groups (Mattioli et al. 1991; Middendorf et al. 1993; Frolov et al. 2010). In my work, the prominence of this shoulder was influenced by the bandwidth of the larger peak, which was correlated with the volume of the side chain at (M)214. RCs with nonpolar (M)214 side chains containing a δ or ε carbon (Leu, Ile, Met) exhibited a more pronounced shoulder than in the (M)L214G/A/C/V RCs (Figure 3.4A). Note that in the case of the (M)L214C and (M)L214V RCs the bandwidth increase of the larger, B_A peak was accompanied by an increase in amplitude, in contrast to the (M)L214A RC, which showed no amplitude increase (also see Figure 3.5). The (M)L214G mutant is a special case, as the significantly broadened B_A/B_B region indicates an additional component at ~795 nm in addition to a large overall decrease in the amplitude of this region relative to the wild type RC (Figures 3.4A and 3.5).

The Q_y transition of the P BChls of nonpolar mutants did not differ greatly. The P BChls in the (M)L214G mutant absorbed slightly to the blue compared to the wild type RC.

The (M)L214A and (M)L214C mutants also exhibited this change, although to a lesser degree than the (M)L214G mutant.



Figure 3.5. Overlay of the $B_{A,B}$ Q_y absorbance band of wild type and (M)L214G/A/C/V mutant RCs from the 11 K absorbance spectra. Spectra were normalized to the maximum absorbance for the dimer (P) BChls. Reprinted with permission from Saer et al. Copyright 2013 American Chemical Society (Saer et al. 2013a).

Turning to the Q_x region, I focus on the overlapping ~595/605 nm peaks, which are composed of contributions from all BChl molecules present in the RC (Shochat et al. 1994). In contrast to the BPhe Q_x region (~535/545 nm), the BChl Q_x region was relatively unchanged (Figure 3.4B). Because the ~595/605 nm peaks were not greatly affected by the nonpolar mutations, relative to the ~535/545 nm peaks, the Q_x absorbance data support the interpretation of the Q_y data, that these mutations did not result in the presence of BChl in the H_A pocket.

3.3.3 The Q_y and Q_x regions in the absorbance spectra of (M)214 polar mutants. The 11 K absorbance spectra of the RC mutants containing a polar residue (i.e. (M)L214 -> H, N, Q) at (M)214 differed significantly from the spectrum of the wild type protein, resembling that of the " β -type" RC mutant (M)L214H (Figure 3.6). For example, in the spectrum of the (M)L214N mutant, a new absorbance band was observed at 785 nm (denoted β), and the absorbance that remained at 756 nm decreased relative to the amplitude of the PA,B absorbance band. In other words, it appeared that the absorbance intensity associated with the Q_y transition of a BPhe group was replaced with the corresponding absorbance of a new BChl group. In addition, there was an increase in the intensity of the B_A BChl absorbance band of the (M)L214H and (M)L214N mutants, where the β and B_A absorbance bands overlap. In the case of the (M)L214Q RC, the relatively small red-shift of the H_A peak resulted in B_A/B_B amplitudes similar to the wild type RC (Figure 3.7). Like the nonpolar and S-containing mutants (except the (M)L214A and (M)L214G mutants), these amplitude increases were also associated with a masking of the B_B should rpresent in the Q_v region of the spectrum.



Figure 3.6. Low temperature (~11 K) steady-state absorption spectra of (A) the Qy and (B) the Qx transitions of the polar (M)214 mutant series. Spectra were normalized to the Q_y absorption maximum of the dimer (P) BChl peaks at ~895 nm. The dashed line indicates the absorption peak wavelength of the BChl Q_x transition in the spectrum of the wild type RC. Reprinted with permission from Saer et al. Copyright 2013 American Chemical Society (Saer et al. 2013a).



Figure 3.7. Overlay of the Q_y transitions corresponding to the B_{A,B} and H_{A,B} regions in the ~11 K absorbance spectrum of the wild type and (M)L214H/N/Q RCs. The peaks denoted " β " correspond to the H_A BChl absorbance in the (M)L214H/N/Q RCs. Spectra were normalized to the maximum absorbance for the dimer (P) BChls. Reprinted with permission from Saer et al. Copyright 2013 American Chemical Society (Saer et al. 2013a).

The Q_x transition spectra of the (M)L214N and (M)L214Q mutants confirm the conclusion that these RCs are β -type (Figure 3.4B). As in the (M)L214H control, the peak around 540 nm was absent from the absorbance spectra of the (M)L214N and (M)L214Q mutants, indicative of a loss of the H_A BPhe from these RCs. There was a corresponding increase in the (M)L214H/Q RC absorbance around 595 nm, indicating the presence of BChl that was absent from the wild type RC.

In the wild type RC the BChl Q_x region is composed of two components: a peak at ~595 nm corresponding to the accessory B_A and B_B BChls, and a shoulder at ~605 nm, which represents the P BChls, as well as a possible contribution from B_A (Shochat et al. 1994; Kirmaier et al. 1985). In the (M)L214H mutant, the BChl Q_x region had an increased amplitude at ~595 nm while retaining the ~605 nm shoulder (compare the relative magnitudes of the ~595 and ~535 nm peaks in the wild type *vs*. the (M)L2124H RC). This Q_x absorbance profile was thought to be representative of the additional BChl, and the corresponding loss of BPhe, at H_A (Kirmaier et al. 1991; Heller et al. 1995). However, in the amide-containing (M)L214N/Q RCs, this ~595 nm peak was blue-shifted relative to the wild type and (M)L214H mutant RCs. The shift was roughly 5 nm (the (M)L214N RC) and ~1-2 nm (the (M)L214Q RC).

In summary, the ground state absorption spectroscopy results suggest that direct ligation of the H_A chlorin to an amino acid side chain is required for the incorporation of BChl into H_A. The use of nonpolar or sulfur-containing moieties at position (M)214 result in the presence of BPhe instead. Based on these findings, it appears that other species, such as water, are unable to act as a fifth ligand, even when a cavity is created in the region corresponding to the center of the chlorin macrocycle, as is the case in the (M)L214G and

(M)L214A mutants. When the H_A chlorin is ligated to a competent residue, the nature of the ligating atom, as well as the volume of the ligating side chain, cause the absorption maximum of the H_A band to shift considerably.

3.4 Functional analysis of (M)214 mutant RCs in photoheterotrophic growth

experiments. The *in vivo* function of (M)214 mutant RCs was evaluated by comparing the RC-dependent phototrophic growth rates of strains containing RC mutations to an otherwise isogenic strain that contains the wild type RC. Under the low photon flux used in this study (5 μ E·m⁻²·s⁻¹), light intensity is a limiting growth factor, and cultures require a significantly longer time to reach stationary phase than when grown under a high light intensity (~500 h *vs.* ~50 h, data not shown). I postulated that under this low light intensity, RC performance *in vivo* may be measured indirectly as a phototrophic growth rate.

As shown in Figure 3.8, the control strain expressing the (M)L214H (β) mutant RC grew much more slowly than the strain containing the wild type RC. It had been reported that the (M)L214H mutant RC is impaired in electron transfer because replacement of the H_A BPhe with a BChl results in an increase in the free energy of the pigment by up to 300 meV, and a lower midpoint potential (Kirmaier et al. 1991; Heller et al. 1995; Fajer et al. 1975; Cotton and Vanduyne 1979). Therefore, my *in vivo* measurement of culture growth rate under low light intensity corresponds to the *in vitro* measurements of electron transfer rates in purified RCs. The growth of strains expressing the (M)L214N or (M)L214Q (β -like) mutant RCs was similarly impaired by comparison to strains expressing the wild type, nonpolar or S-containing (M)214 mutants. These results indicate that the (M)L214H RC.

In contrast to the growth of the polar mutants, all of the strains containing nonpolar or S-containing substitutions at (M)214 grew with approximately the same kinetics as the wild type strain (Figure 3.8). The average growth of the nonpolar or S-containing mutants (0.5 Klett units per hour, Appendix 1) was faster than that of the polar mutants (0.1 Klett units per hour, Appendix 1). The similar growth kinetics of the nonpolar or S-containing mutants was surprising, specifically because the (M)L214G/A mutants were found to be impaired in electron transfer from $H_{A^-} \rightarrow Q_A$, such that charge recombination competes with the forward reaction, resulting in a drop in the overall yield of charge separation (Pan et al. 2013). These contrasting *in vitro* results are considered in more detail in Section 3.6.



Figure 3.8. Photoheterotrophic growth of *R*. *sphaeroides* strains containing mutant RCs cultured with 5 μ E.m⁻².s⁻¹ illumination. The $\Delta puhA$ mutant, which lacks the RC H protein illustrates zero-growth. WT, wild type RC; single letter amino acid code identifies the residue at (M)214 in the RC mutants. Curves are representative of three independent measurements. Reprinted with permission from Saer et al. Copyright 2013 American Chemical Society (Saer et al. 2013a).

3.5 X-ray crystallography of (M)L214G, (M)L214A, and (M)L214N mutant RCs.

The (M)L214G, (M)L214A and (M)L214N mutants exhibited notable differences in their low-temperature absorbance spectra compared to the wild type RC and to each other (Figures 3.4 and 3.6). In order to identify structural changes in the RC, such as changes in the positions of H_A and B_A, that might account for these spectroscopic changes, the crystal structures of these three mutant RCs were determined to resolutions of 2.20 Å, 2.70 Å, and 2.85 Å, respectively (see Appendix 1). The protein backbones of all three mutant RCs were almost identical to that of the wild type protein (PDB: 2J8C, RMSDs < 0.15 Å for all CA atoms).

In the crystal structures of the (M)L214G and (M)L214A mutant RCs, no difference from the wild type RC in the location of the H_A BPhe was observed within coordinate error. The H_A chlorin was well-defined but lacked electron density indicative of a metal chelated in the macrocycle, consistent with the interpretation that the H_A pocket contains a BPhe.

However, there were clear differences in electron density in the vicinity of (M)214 in addition to the mutations themselves. These differences were clearest in the highest resolution structure of the (M)L214G mutant RC. In this structure, electron density around the phytyl tail of B_A was discontinuous in the region where the tail bends, near the C4 methyl group (Figure 3.9A), implying that the B_A tail is disordered. Notably, the bend in the phytyl tail is in close proximity to residue (M)L214 in the wild type RC (Figure 3.9B). The volume made available by the deletion of the leucine side chain in the (M)L214G mutant RC is occupied in part by the rotation around the phytyl tail bend. By inspection of difference omit maps, the B_A phytyl tail was modeled in two conformations with equal occupancy. The "right" conformer depicted in Figure 3.9A is equivalent to the conformation observed in the
wild type *R. sphaeroides* RC (Figure 3.9B). In the "left" conformation, also shown in Figure 3.9A, the B_A phytyl tail is directed away from the H_A macrocycle such that a portion of H_A ring 1 is exposed to the solvent (see Discussion). In this conformer, the phytyl chain and a glycerol (cryoprotectant) molecule displace the molecule of LDAO detergent that occupies this space in the structure of the wild type RC (compare Figures 3.9A and 3.9B). The two conformers and glycerol molecule do not completely account for the difference density present in sigma weighted and unweighted maps at the mutation site or in the region of the two phytyl tail conformations, suggesting that partially occupied solvent molecules and perhaps additional conformations of the phytyl tail are present. The difference maps derived from the (M)L214A crystal diffraction data are consistent with the two conformations of the phytyl tail (Figure 3.9C), although the lower quality of the (M)L214A data restrict structural comparisons with the (M)L214G structure to the mutation site.



Figure 3.9. Omit difference electron density maps of: (A), the (M)L214G RC; (B), the wild type RC (PDB:2J8C); and (C), the (M)L214A RC. These maps were generated by setting the occupancy of the phytyl tail and the nearby LDAO (if present) to zero, followed by 8 cycles of refinement. Discontinuous density is visible in the "right" conformation of the (M)L214G mutant, and continuous density for the "left" conformation can also be seen (A, green mesh). Electron density meshes are contoured at 3.0σ and carved in a 2.5 Å radius. Oxygen atoms are colored red and nitrogen atoms blue. Carbon atoms are colored orange for LDAO, grey for glycerol and H_A, yellow for residue (M)214, and green, cyan, and magenta, respectively, for the (M)L214G, wild type, and (M)L214A RCs. Reprinted with permission from Saer et al. Copyright 2013 American Chemical Society (Saer et al. 2013a).

In the (M)L214N crystal structure, positive difference electron density was obvious at the center of the chlorin macrocycle in the H_A position (Figure 3.10). This density was modeled as a Mg^{2+} ion, confirming that the chlorin at this position is a BChl group, in agreement with the results obtained in Table 3.1. After subsequent refinement, the B-factor associated with the Mg²⁺ ion was commensurate with the BChl ring, and no residual difference density was present. At residue (M)214, adjacent to the Mg²⁺, density for an Asn side chain was present and modeled accordingly. The (M)N214 OD2 atom is located approximately 2.0 Å from the Mg^{2+} , the Mg^{2+} to OD2 bond angle is approximately perpendicular to the plane of the macrocycle, and the Mg^{2+} ion is displaced ~0.4 Å from the plane formed by the tetrapyrrole N atoms towards the new ligand, supporting the assignment of a metal-ligand bond. A similar ~0.4 Å displacement of Mg²⁺ from the plane was observed in the equivalent N to Mg^{2+} bond between (L)H153 and the B_A BChl (not shown). The ND1 atom of (M)N214 is within H-bond distance (3.1 Å) of the main chain carbonyl of (M)Y210. The alternative amide conformation of the Asn side chain with Mg^{2+} coordination by the ND1 atom of Asn214 is unlikely because of the inability of the OD2 to establish a hydrogen bond with this main chain carbonyl group, and because of the requirement for the metal to displace a proton of the NH₂ group to form a metal-N ligand. Furthermore, the lone electron pair of the amide nitrogen atom should participate in a resonance structure of the amide group, resulting in a partial positive charge on the nitrogen.



Figure 3.10. Omit difference electron density map of the (M)L214N RC, illustrating positive difference density for the Mg^{2+} ion at the center of the chlorin ring of BChl in the H_A site. The electron density mesh is contoured at 4.0 σ and carved in a 2.0 Å radius. Oxygen atoms are colored red and nitrogen atoms are in dark blue. H_A carbon atoms are in lighter blue and (M)L214N carbon atoms are shown in yellow. Reprinted with permission from Saer et al. Copyright 2013 American Chemical Society (Saer et al. 2013a).

The results from section 3.5 suggest that the protein component of the RC is largely undisturbed from mutation of the (M)214 residue to amino acids containing small, nonpolar side chains. In contrast to the protein component, the phytyl tail of the nearby BChl residue, B_A, appears to be disordered, as demonstrated by the electron density maps (Figure 3.9). Furthermore, the structure of the (M)L214N mutant revealed that amide-containing side chains are indeed capable of acting as fifth coordinates to the H_A chlorin. I suggest that this ligating residue is the amide oxygen, and not the nitrogen.

3.6 Kinetic analysis of wild type and mutant RCs with nonpolar or sulfur-

containing side chains at (M)214. Transient difference spectroscopy was used to evaluate the *in vitro* function of the (M)L214 nonpolar or S-containing mutant RC series on several levels, including the primary events of charge separation, the subsequent electron transfer steps, as well as the quantum yield of charge separation (calculated within a 6 ns time window). In this form of spectroscopy, the obtained data are interpreted as 2D datasets, representing a set of difference spectra (excited minus non-excited) spanning from 500 nm to 960 nm, measured over a time window from 150 fs (0.15 ps) to 6 ns (6000 ps). Thus, data can be interpreted as changes in absorbance (differences) measured at a single wavelength over time (a kinetic trace), or entire difference spectra displayed at single points in time (transient absorbance spectra). One method of further analyzing such spectra is to perform what is called a global analysis. In this technique, the datasets are modeled to fit a series of exponential decays using a least squares approach, with the time constant of each decay being representative of the disappearance of an entire transient difference spectrum. The values of the time constants signify the point in time in which the transient difference spectrum has decayed to 1/e (~36.8%) of its maximum value. These transient difference

spectra have been called "decay associated difference spectra" (van Stokkum et al. 2004). The total number of decay associated difference spectra present in a dataset is equal to the number of exponentials used to fit the dataset. The sum of all decay associated difference spectra at a time point that corresponds to the time constant of only one of these spectra is termed the "evolution associated difference spectrum" (EADS (van Stokkum et al. 2004)). When referring to the RC, the different EADSs are by no means representative of the different electronic states of the protein, however they provide a simple method of visualizing the temporal progression of the difference spectra dataset. When the different EADSs of the wild type protein are calculated, a meaningful comparative analysis can be made to mutant RCs, providing insight into the electron transfer behavior of these proteins in the ultrafast time domain.

Shortly following excitation of the RC sample (0.5 ps post excitation), the transient absorbance spectrum becomes dominated by the excited state special pair, or P*. This spectrum is characterized by a large broadband bleaching (negative amplitude) of the spectral region between 800 nm to 950 nm, and represents a large degree of stimulated emission from the excited state. The decay of this stimulated emission indicates a decrease, but not a complete disappearance, of the bleaching amplitude around 800 nm to 950 nm. Because other transient signals of the RC (such as P⁺) eventually overlap with this bleaching signal within a few picoseconds, the decay of stimulated emission from P* is commonly measured as a kinetic trace from 925 nm to 930 nm, because stimulated emission represents the only contribution to this spectral region (note how the bleaching in this region disappears after 10 ps in the inset of Figure 3.11). Because the rate of non-productive fluorescence (*i.e.*, fluorescence emission in the absence of electron transfer) from P* occurs on a timescale of

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~500 ps (Jones 2009), any observed changes in P* stimulated emission within 10 ps result from direct electron transfer from P* to a nearby cofactor, as seen in the wild type RC where P* almost always decays via forward electron transfer (Figure 3.11).



Figure 3.11. Normalized kinetics of stimulated emission decay in wild type and (M)L214 mutant RCs measured at 926 nm with an excitation pulse at 865 nm. Letters in the legend represent: WT, wild type; LM, (M)L214M; LC, (M)L214C; and so on. The inset shows the transient absorption difference spectra of the wild type RC 0.5 ps (white circles) and 10 ps (red circles) post excitation. The upward pointing arrows are representative of wavelengths in which kinetic traces have been described in Section 3.6 of this work. Reprinted with permission from Pan et al. Copyright 2013 American Chemical Society (Pan et al. 2013).

The rate of stimulated emission decay at 926 nm from RCs containing Cys, Met, Ala, or Gly at (M)214 closely resemble that of the wild type RC, in that the signal decays almost completely within 10 ps post excitation. Thus, it appears that these RCs are able to mobilize an electron from P* to a nearby cofactor just as efficiently as the wild type RC.

Following the oxidation of P*, the RC enters a series of charge-separated states via electron transfer reactions along the A branch, increasing the distance between P⁺ and its electron. The nearest cofactor to P⁺ is B_A, which is reduced with a time constant of ~3 ps post excitation. This P⁺B_A⁻ state quickly decays to P⁺H_A⁻ with a time constant of ~1 ps, The speed at which P⁺B_A⁻ decays into P⁺H_A⁻ made the detection of the B_A⁻ anion difficult, with some researchers even questioning its presence (Martin et al. 1986), Nevertheless, spectroscopic studies in the near IR region have shown that B_A is a genuine electron carrier with a spectroscopic transient at 1020 nm (Arlt et al. 1993). Because my work did not capture data on wavelengths to the red of 960 nm, the kinetics of B_A⁻ are excluded from the study. Instead, I focus on the formation of H_A⁻, which has two characteristic transient signatures. The first transient signature is an electrochromic shift of the ground state absorbance of B_A upon the RC entering the P⁺H_A⁻ charge separated state. Specifically, the 802 nm absorbance band of B_A shifts to a higher energy. Measurement of this shift is observed at 785 nm (Figure 3.12).



Figure 3.12. Normalized kinetic traces of the electrochromic shift of the BA absorbance, measured at 785 nm, for wild type and (M)L214 mutant RCs. Solid lines are fits to the data points (circles). Reprinted with permission from Pan et al. Copyright 2013 American Chemical Society (Pan et al. 2013). LC = (M)L214C, LA = (M)L214A, LG = (M)L214G. The horizontal black line is a zero absorbance reference line. Data points to the left of the vertical black line are on a linear time scale, and those to the right of the line are on a logarithmic time scale.

Within 0.5 ps, there was a sharp absorbance increase at 785 nm in all RCs measured (the (M)L214M kinetics were practically identical to wild type, and are thus not shown in Figure 3.12 or any subsequent kinetic traces). This increase represents a broadband absorption increase due to formation of P* (Martin et al. 1986). Following this increase, the amplitude of the absorbance in this region continued to increase, which is attributed to a shift from the ground state absorbance of B_A due to the emergence of the H_A anion. Interestingly, the amplitude of this absorbance increase is proportional to the volume of the side chain at (M)214, such that larger side chain volumes are associated with a larger amplitude increase at 785 nm. Although the cause of these amplitude differences is not clear, it is possible that a higher degree of static heterogeneity (i.e. multiple subpopulations of RCs in a single sample) present in the RCs with lower (M)L214 side chain volumes accounts for this difference. This is because the increased bandwidth of an absorption band, arising from static heterogeneity (most evident in the B_A absorption band in the Q_y transition of the (M)L214G mutant), concurs with a decrease in peak amplitude. Nevertheless, it is clearly evident from the amplitude increases at 785 nm that the $P^+H_A^-$ state is attained in all the measured RCs.

The second spectral signature that is characteristic of the H_{A} anion is an increase in absorbance at 675 nm. Although this signal is broadband, detection at 675 nm has been classically used in the literature as the wavelength of measurement. The kinetic traces measured at 675 nm for the wild type and RCs containing Cys, Ala, or Gly at (M)214 are shown in Figure 3.13.



Figure 3.13. Normalized kinetic traces measured at 675 nm for wild type and (M)L214G/A/C mutant RCs. A quinone-lacking RC (WT-deQ) is used as a negative control. Reprinted with permission from Pan et al. Copyright 2013 American Chemical Society (Pan et al. 2013). The vertical black line represents the shift from a linear to logarithmic time scale. The horizontal black line is a zero ΔA reference line.

In Figure 3.13, the absorbance amplitude at 675 nm increases to a maximum within 10 to 20 ps post excitation. In my work, we assumed that the majority of the population of RCs in a sample were capable of achieving the charge separated $P^+H_A^-$ state. Thus, all spectra in Figure 3.13 were normalized to the Q_x bleaching of P at 600 nm at 0.5 ps post excitation. In the wild type RC, this 675 nm absorbance signature decays with a time constant of approximately 200 ps, which is indicative of forward electron transfer to the primary quinone, Q_A. In the mutant RCs, however, one can see that this increase in amplitude decays with kinetics different from the wild type RC. Specifically, the decay time is increased to the point where none of the RCs with small, nonpolar or S-containing moieties at HA ((M)L214G/A/C) show signs of complete decay within the 6 ns time window of measurement. In addition, the 675 nm transient decay kinetics of the (M)L214G/A/C RCs require multiple exponential components to fully fit to the data (compared to a single exponential in the wild type RC). My interpretation is that there exists a sub-population of these three mutant RCs with a long-lived $P^+H_A^-$ state. This means that either: (i) the forward electron transfer event in the (M)L214G/A/C RCs occurrs with more than one rate; (ii) the $P^+H_A^-$ state begins to decay via the non-productive pathway of charge recombination, resulting in a return to the ground state; or (iii) both i) and ii) happen at the same time. To begin to parse this issue, the kinetics of ground state recombination was measured. Figure 3.14 compares the normalized kinetics of wild type and (M)L214G/A/C RCs at 837 nm. Because the negative bleaching amplitude from P⁺ dominates the absorption changes at this wavelength, a decrease in bleaching amplitude at 837 nm after ~15 ps can be attributed to charge recombination of P+ from some charge separated state, whether it is $P^+B_A^-$, $P^+H_A^-$, or a combination of both states.

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Figure 3.14. Normalized kinetic traces (circles) and their respective fits (solid lines) measured at 837 nm for wild type and (M)L214G/A/C mutant RCs. A quinone-lacking RC is used as a negative control. Black circles: wild type, blue circles: (M)L214C, teal Circles: (M)L214A, pink circles: (M)L214G, white circles: quinone-removed RCs. The vertical black line represents the shift from a linear to logarithmic time scale. The horizontal black line is a zero ΔA reference line. Reprinted with permission from Pan et al. Copyright 2013 American Chemical Society (Pan et al. 2013).

It is clear from Figure 3.14 that the wild type RC does not exhibit charge recombination on this time scale because, after the initial bleaching from sample excitation, there was no decay of bleaching throughout the window of measurement. In contrast, the bleaching of the (M)L214G/A/C RCs decays in the nanosecond time range. The degree of decay was most pronounced in the (M)L214G mutant, where the curve decayed almost halfway within the measurement window, similar in magnitude to an RC in which the Q_A quinone was removed by chemical means. By comparison of the bleaching kinetics, the behaviour of RCs with small, nonpolar or S-containing moieties at H_A in the nanosecond time domain becomes clearer; although some forward electron transfer occurs on the hundreds of ps time scale, the population of RCs with long-lived P⁺H_A⁻ states is poised in a state of competition between a slow forward electron transfer and charge recombination. The degree of forward electron transfer *vs*. charge recombination appears to be correlated with the volume of the (M)214 side chain, because the (M)L214G mutant exhibited the largest amount of recombination (Figure 3.14).

The similar recombination kinetics of the (M)L214G and the Q_A-lacking RCs led me to investigate the possibility that some of the Q_A quinones were absent in this RC sample. To address this, a saturated solution of ubiquinone-10 in ethanol was added to both the wild type RC and the (M)L214G RC at 1/10 the sample volume. The RCs were left to incubate for 1 hour in the dark at room temperature and an absorption spectrum was measured. Because the addition of quinone solution changed the baseline of the absorption spectrum for both wild type and (M)L214G RCs, a light minus dark difference spectrum was taken for each sample before and after the addition of quinone. Any increases in quinone occupancy were interpreted as increases in bleaching amplitude at 865 nm. The spectra were not normalized, however the samples were maintained at the same concentration (42 μ M). The results, shown in Appendix 5, show no major changes in the difference spectra in both the wild type and (M)L214G RCs before or after the addition of ubiquinone. Thus, it appears that the (M)L214G sample is Q_A-replete. These data are in agreement with our crystallographic data for the (M)L214G RC, which show no significant deviations in B-factors for Q_A head atoms (~31-39) compared to the rest of the RC (overall B-factor is 40, the tail atoms have higher Bfactors, but this is feature common to all RC crystallographic datasets).

In contrast to the *in vivo* photoheterotrophic growth experiments, the results from the work in this section suggest that RCs with smaller amino acids at (M)214 are associated with more complex kinetics, as well as a lower degree of charge separation ability. These RCs are unable to maintain the ~100% degree of charge separation observed in the wild type RC over a 6 ns time window. Furthermore, the decay of the $P^+H_A^-$ state appears to be multiexponential in the (M)L214G/A/C mutants. An explanation of these kinetics is given in the Discussion.

3.7 Pigment substitutions of wild type and (M)L214G RCs. When heated in the presence of a molar excess of a heterologous chlorin-type of pigment, the *R. sphaeroides* RC has been shown to substitute native pigments for a heterologous pigment. Such experiments have demonstrated selectivity in the binding pockets of the RC, because only certain heterologous pigments substitute for the native pigment in only certain RC pockets. These experiments have also provided insight into the effects of pigment substitution on neighboring chromophores (Scheer and Hartwich 1995), as well as the effects of substituted pigments on the electron transfer properties of bacterial RCs (Huber et al. 1995). In my

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research, pigment substitution experiments were used to investigate the role of (M)214 in pigment selectivity by comparing the results obtained from wild type and (M)L214G RC pigment substitutions. I investigated several temperatures, lengths of time, and three pigments were used in substitution experiments: BChl, plant Pheo, and plant Chl. Both plant Chl and Pheo differ from BChl and BPhe in that they contain a vinyl group instead of an acetyl group on ring A, as well as an additional double bond on ring B. Plant Pheo differs from Chl in that, like BPhe it does not contain a central Mg²⁺ ion. The results were analyzed using room temperature absorption spectroscopy, and some are depicted in Figure 3.15.



Figure 3.15. Absorption spectra of wild type (left) and (M)L214G (right) RCs after incubation at 37° C with a molar excess of heterologous pigment. The spectra were normalized to the maximum absorbance of P. The absorbance peaks are labeled according to the convention in Figure 1.3. The Chl/Pheo peak corresponds to the absorption band of plant chlorophyll *a* and pheophytin *a*.

My work on substitution of wild type and (M)L214G RCs with plant Pheo demonstrates that chromophore substitution occurs at 37°C, although the substitution is by no means complete when compared with previous results for the WT RC, in which the incubation temperature was 42.7 °C (Franken et al. 1997). Nevertheless, the amount of substitution was sufficient for a comparative analysis using absorbance spectroscopy. The absorbance spectra of wild type and (M)L214G RC substituted with Pheo show a decrease of absorbance amplitude in the 760 nm band, with an associated increase in 670 nm absorbance. Both RCs appear to show an equal degree of substitution, although the shapes of the peaks were different. The 670 nm peak in the Pheo-substituted wild type RC contained a small shoulder on the red side. In addition, the 760 nm peak in the (M)L214G mutant RC appeared as a shoulder to the blue of the $B_{A,B}$ peak, lacking a discrete apex. Although the shapes of both the 760 nm and 670 nm peaks differ between the two RCs, I suggest that these changes are a consequence of the different binding pocket environments induced by the amino acid change. The amplitudes of both the 670 nm peak and 760 nm peak are roughly equal between the wild type and (M)L214G RC. Specifically, the broadened B_{A,B} absorption band of the (M)L214G RC (see Section 3.3) allows for a larger degree of overlap between this band and the smaller neighboring $H_{A,B}$ band, thereby making this peak appear more like a shoulder when its amplitude was decreased due to the (M)L214G substitution (Figure 3.15). The shoulder of the Pheo band observed in the wild type RC is more difficult to explain, as the absorbance properties of Pheo in bacterial RCs is poorly understood. However this shoulder may be the result of a smaller fraction of Pheo incorporating into H_A compared to H_B. In the low temperature absorbance spectroscopy results (Fig 3.4), the H_A Q_x transition band is redshifted relative to the H_B band, and so if the same effect were to occur when Pheo is inserted

into the H_A pocket this shoulder would represent the population of Pheo present at H_A . In the (M)L214G RC the spectral shifting of the Q_x transition is less pronounced (Fig 3.4), and therefore a shoulder on the 670 nm peak would not be expected.

The use of BChl *a* in substitution experiments on the wild type RC yielded no discernible changes in the absorbance spectrum, as shown in Figure 3.15. In contrast, the (M)L214G mutant RC produced a different spectral phenotype post-substitution. Firstly, the amplitude of the ~760 nm H_{A,B} peak in the BChl *a*-substituted (M)L214G RC was decreased relative to the nonsubstituted RC (Figure 3.15). This was accompanied by an increase in absorbance in the region corresponding to the trough between the ~760 nm H_{A,B} peak and the ~802 nm B_{A,B} peak. Taken together, these results indicate that a spectral red-shift at one of the chromophores in the (M)L214G RC resulted from pigment substitution using BChl *a*. Thus, it appears that incubating the (M)L214G with a molar excess of purified BChl *a* results in the incorporation of the metal-containing chlorin BChl *a* in this H_A pocket, although further experiments are required to provide a more definitive answer.

Because the absorption spectrum of the Q_y transition of free BChl *a* overlaps with that of the RC to a large degree, I sought to perform a similar pigment substitution experiment using a chromophore that does not spectrally overlap the absorption bands of the RC. It was thought that this approach might paint a clearer picture with regards to the H_A pocket differences between the (M)L214G and wild type RC. Thus, the wild type and (M)L214G RC were used in substitution experiments employing the heterologous pigment Chl *a*. The Q band of free Chl *a* is similar to that of Pheo in the sense that it absorbs maximally at ~675 nm, in a region where the RC absorption bands exhibit no major contributions. Like the experiments with plant Pheo, I would expect the outcome of a successful substitution with

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Chl *a* to result in both a decrease in one of the Q_y absorption band of the RC, as well as the presence of a new absorption band near ~675 nm since the Q band of Chl a peaks at a shorter wavelength than the Q_y band of BChl or BPhe. The results of pigment substitution with Chl a are also shown in Figure 3.15. In the wild type RC, the Q_y transition of the RC absorption spectrum remained largely unchanged, although there was a new peak at ~675 nm present. This result was not expected because the appearance of a new absorbance band without an accompanying decrease in the intensity of one of the RC absorbance bands suggests that pigments were binding to the sample without substitution. Subsequent re-purification of the RC using DEAE anion exchange chromatography failed to remove this peak from the spectrum. Because there were no changes to the three Q_y peaks of the RC spectrum, it appears that Chl a binds the RC in a non-specific manner more tightly than the other tested chromophores (Pheo and BChl a). Repeating this experiment, but now with the (M)L214G RC produced an absorption spectrum that was largely similar to the Chl *a*-substituted wild type RC, although there was a noticeable decrease in ~760 nm peak amplitude. In addition, the amplitude of the ~ 802 nm B_{A,B} peak decreased. Although this decrease could be in part a result of the spectral overlap between the ~760 nm and ~802 nm peaks in the RC absorption spectrum, I do not believe that this explanation is sufficient to account for the entire ~802 nm absorbance decrease. This is because the experiment with Pheo substitution produced a \sim 760 nm peak with a peak amplitude similar to that of the Chl a-substituted (M)L214G RC, whereas the ~802 nm peak did not decrease by a similar amount. Thus, I suggest that in the Chl *a* substitution experiment on the (M)L214G RC the B_A site was affected. Because the ~760 nm peak represents contributions from BPhe in the $H_{A,B}$ pockets, it could be that the pigments at H_{A,B} are either removed from the RC or are substituted with Chl a in these

experiments. Because the process of heating the (M)L214G RC does not result in loss of pigments, as evidenced by a lack of change in the RC absorption spectrum, the latter explanation (that Chl *a* is incorporated into the H_{A,B} pockets) seems more likely. Taken together, the experiments described above illustrate that a bulky residue like Leu at position (M)214 in the RC plays a role in the structural integrity and pigment specificity of this pigment-protein complex. Changing this side chain to a smaller moiety appears to allow a wider variety of heterologous chlorins, including those that contain central metal atoms, to incorporate into the RC under conditions of prolonged exposure to higher temperatures.

The above results hint at a novel role for residue (M)214 in providing pigment selectivity at the H_A site. In contrast to the wild type RC, whose Q_y absorption spectrum remains largely unchanged after incubation with a molar excess of either Chl a or BChl, the (M)L214G mutant does incur spectral changes. These changes are localized on the Q_y absorption band of the RC BPhes, and suggest that the (M)L214G RC is indeed capable of accepting metallo-(bacterio)chlorins in the H_A pocket.

3.8. Bacteriopheophytin incorporation into the RC in the presence of

adventitious ligands. The results of pigment substitution experiments on the wild type and (M)L214G mutant RCs appeared to complicate the issue of the origin of BPhe, in addition to the question of H_A pocket selectivity in the RC. If metal-containing chlorins were incorporated into the (M)L214G mutant *in vitro*, then why did purification of this RC not yield an appreciable population of RCs with a molecule of BChl at H_A ? One possible answer is that BChls may bind to an axial ligand during the folding of the RC and, if such a ligand is not present, the Mg^{2+} ion leaves the chlorin macrocycle. Thus, in order to test whether the wild type or (M)L214G mutant RC can accommodate a molecule of BChl at H_A *in vivo*, these

two RCs were purified in the presence of an adventitious ligand which could stabilize the BChl molecule in a pentacoordinate state during the RC folding process. Previous reports have shown that imidazole can provide such a function in both heme-containing proteins, as well as the RC (Depillis et al. 1994; Goldsmith et al. 1996). Because it was reported in Goldsmith et al. that concentrations of imidazole in excess of 5 mM produced inhibitory effects on cell growth (Goldsmith et al. 1996), this concentration was chosen as a starting point for the subsequent experiments. In order to prevent dilution of the solute until the final purified protein was obtained, imidazole was added to all the buffers used in the RC purification procedure, at a final concentration of 5 mM. Interestingly, the concentration of imidazole used (5 mM) proved to be inhibitory for culture growth when cells were grown under chemotrophic conditions (aerobically in the absence of illumination). Under photoheterotrophic conditions (anaerobically with illumination), however, the cultures appeared to be tolerant of this concentration of imidazole however they required several more days to reach stationary phase. This was undesirable because the extra growth time may give rise to faster-growing spontaneous mutants. It was thus necessary to find the maximum allowable concentration of imidazole that was permissive for chemotrophic growth in the dark. R. sphaeroides strain \triangle RCLH containing plasmid pRS1 was grown in RLB media containing concentrations of imidazole ranging from 1 to 5 mM, and optical density readings were obtained after 72 hours. The results of this experiment are shown in Figure 3.16. Under conditions of chemotrophic growth in the dark, the results from Figure 3.16 suggest that R. sphaeroides loses its ability to grow to an appreciable culture density when the concentration of imidazole in the medium is greater than 2 mM. This concentration was thus used to for a

comparative analysis of RCs purified from cultures grown with or without imidazole in the medium.



Figure 3.16. Optical density of chemotrophically grown *R. sphaeroides* strain Δ RCLH containing plasmid pRS1 under varying concentrations of imidazole. Measurements were taken at 700 nm after 72 hours of growth at 30° C in the dark. Error bars represent standard deviations about the mean from triplicate measurements.

Following the determination of the maximum allowable concentration of imidazole that was permissive of chemotrophic growth, the wild type and (M)L214G RCs were purified from cultures grown in the presence of 2 mM imidazole. Figure 3.17 shows an overlay of the wild type and (M)L214G RC absorption spectra after purification with or without the addition of 2 mM imidazole to the growth medium and buffers used for purification.



Figure 3.17. Absorbance spectra of wild type and (M)L214G RCs from cultures grown photoheterotrophically in the presence or absence of 2 mM imidazole, and purified in the presence or absence of 2 mM imidazole. The four spectra were normalized to the maximum absorbance of the $P_{A,B}$ band at ~865 nm. The absorbance peaks are labeled according to the convention in Figure 1.3.

The results obtained from imidazole-purified RCs demonstrate that there was little to no difference in the absorption spectra of the wild type RC from cultures grown chemotrophically in the presence or absence of 2 mM imidazole, and purified in the presence or absence of imidazole. The absorption spectra of the two wild type RC samples almost overlap throughout the entire Qy transition region, and so I conclude that imidazole does not have an effect on the pigment composition of this RC.

The (M)L214G RC, on the other hand, appears to have been affected in cofactor composition when imidazole was added to the growth medium. Specifically, the Qy transition bands corresponding to the $B_{A,B}$ and $H_{A,B}$ chromophores of the imidazole-purified (M)L214G RC showed decreases in amplitude relative to that of the (M)L214G RC purified in the absence of this solute, indicating that one or more cofactors were not present in the (M)L214G RC purified with imidazole. The Q_x transition also exhibits an absorption intensity decrease, however the decrease is localized on the Q_x absorption of the BPhes alone. Pigments were then extracted from the purified RCs and the BChl:BPhe ratio was calculated according to the procedure outlined in the Materials and Methods. The calculated BChl:BPhe ratios are listed in Table 3.2. The results shown in Table 3.2 reveal that the BChl:BPhe ratio of the (M)L214G RC purified in 2 mM imidazole was close to 3.3, whereas the ratios for the other three measured samples were between 2 and 2.6.

Table 3.2. Calculated BChl:BPhe ratios of wild type and (M)L214G RCs purified in the

RC	BChl:BPhe Ratio*		
wild type	2.03 ± 0.09		
wild type + Imidazole	2.29 ± 0.01		
(M)L214G	2.60 ± 0.11		
(M)L214G + Imidazole	3.27 ± 0.02		

presence and absence of 2 mM Imidazole

* Values are averages of three extractions \pm the standard deviation about the mean.

Taken together with the results from Figure 3.17, the data in Table 3.2 indicate that one of the BPhe molecules was partially absent from the imidazole-purified (M)L214G RC. Thus, it appears that the (M)214 residue plays a role in the efficient incorporation of pigments into the RC *in vivo* when solutes which can act as adventitious ligands are present in the growth medium. A detailed interpretation of these results is presented in the Section 4.1 of the Discussion.

3.9 Structural, spectroscopic, and functional studies of RCs containing zincbacteriochlorophyll (Zn-BChl) in place of both BChl and BPhe. The results

obtained from Section 3.6 highlighted an important role for residue (M)214 in electron transfer by showing that changes in the protein environment near the photoactive H_A cofactor of the RC changed the rate of electron transfer without affecting the cofactor composition. In Sections 3.7 and 3.8, the effects of cofactor substitution and growth in the presence of adventitious ligands on the wild type and (M)L214G mutant RCs are described. In related research, the electron transfer kinetics of RCs containing Zn-BChls (in place of BChls and Bphes) were studied.

The initial research demonstrated that an otherwise wild type RC containing six Zn-BChls in place of the four BChls and two Bphes of the wild type RC was capable of charge separation with yields similar to the wild type RC (Lin et al. 2009). This was surprising, especially considering the fact that a Zn-BChl was present as the H_A cofactor. The reason this was surprising was because of the observed low charge separation yields of the (M)L214H RC, which, like the Zn-RC studied by Lin *et al.*, also contained a metallo-chlorin at H_A (Kirmaier et al. 1995a, b; Kirmaier et al. 1991). Lin *et al.* postulated that electron transfer through H_A is strongly affected by the coordination state, and less so by the presence or absence of a metal

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center in the chlorin ring. The results from Section 3.6, however, show that the protein component of the RC plays a role in controlling electron transfer rates irrespective of the H_A coordination state, because of the kinetic disparity observed between RCs with identical pigment compositions and ligation states. To gain a more comprehensive understanding of the different factors that govern electron transfer rates through H_A , I used transient spectroscopy to compare RCs containing six Zn-BChls, in which the coordination state of the Ha Zn-BChl was changed from four to five by changing (M)L214 (denoted the Zn-RC) to His (denoted the Zn- β -RC). These two types of Zn-RC were compared to the wild type RC and the (M)L214H mutant. Using these comparisons, I attempted to parse the electron transfer effects related to coordination state of the H_A cofactor from those related to the presence or absence of a metal center at H_A.

From my analysis of the measurements of electron transfer kinetics and the corresponding structural data of Zn-BChl- containing RCs I suggest that the coordination state of a chlorin cofactor in a protein governs the redox midpoint potential, and therefore the electron transfer properties in the RC protein.

3.9.1 Comparison of the wild type RC, the (M)L214H RC, the Zn-RC, and the Zn-\beta-RC in low temperature absorption spectroscopy. The ground state absorption spectra of the wild type, (M)L214H mutant, Zn-RC and Zn- β -RC at 10 K are shown in Figure 3.18. The spectra were normalized to the amplitude of the Q_y band of the dimeric BChls (P_{A,B}). The Q_y transition differences in absorption between the Zn- β -RC and the Zn-RC are more subtle than the differences between the (M)L214H mutant and the wild type RC (described in Section 3.3.3). The P_{A,B} band in the Zn- β -RC is slightly broadened relative to the Zn-RC, and the B_{A,B} and H_{A,B} Q_y bands of the Zn- β -RC appear to be shifted ~2 nm to the blue. In the Q_x

transition region of the Zn- β -RC spectrum the conversion of the 4-coordinate Zn-BChl at H_A in the Zn-RC to 5-coordinate in the Zn- β -RC is indicated by the increased amplitude of the ~590 nm peak, corresponding to a 5-coordinate Zn-BChl, accompanied by the disappearance of a peak at ~560 nm, where the 4-coordinate Zn-BChl absorbs in the Zn-RC (Lin et al. 2009; Neupane et al. 2012). The H_B Zn-BChl content in the Zn-RC appears to be low, because the peak at ~560 nm seems too small to represent contributions from two Zn-BChls (compare this region in the Zn-RC to the wild type RC, which shows two distinct peaks of roughly the same size). In the Zn- β -RC, this the ~560 nm peak is almost completely absent, suggesting that little to no 4-coordinate Zn-BChls are present in this RC. This is in contrast to the (M)L214H mutant, in which the corresponding BPhe peak at ~530 nm is still present despite the absence of one of the BPhe peaks.



Figure 3.18. Ground state absorption spectra obtained at 10 K for (A) the wild type (WT, black), and (M)L214H mutant RCs (green), and (B) the Zn-RC (red) and Zn- β RCs (blue). The spectra were normalized to the P865 absorption maximum. Peaks are labeled according to the convention on Figure 1.3. The peak denoted " β " refers to the H_A BChl in the (M)L214H mutant. Tetra- and Penta- Zn-BChl peak labels refer to the absorption bands of 4- and 5-coordinate Zn-BChl, respectively The spectrum of the Zn-RC was corrected for scatter by subtracting a straight line from 500 to 730 nm. Figure modified with permission from Saer et al. (Saer et al. 2013b).

The bandwidth of $P_{A,B}$ absorption in the Q_y transition for both the (M)L214H and Zn- β -RC appears slightly broadened on the shorter wavelength side relative to their non- β counterparts. Therefore, although (M)H214 should not be in the immediate vicinity of the macrocycle component of the dimeric (Zn-)BChls, it appears that this change slightly perturbs this region. The highest resolution crystal structure available for the RC shows that (M)L214 lies close to the phytyl tail of the P_A BChl (Koepke et al. 2007). Thus, it appears that this bandwidth increase may be a consequence of a direct interaction between the histidine residue at (M)214 and the P_A tail, or an indirect effect. Alternatively, the (M)L214H mutation may induce a perturbation in the membrane-spanning helix (helix D) in which this residue resides. Because this helix also contains residue (M)H202, which acts as the fifth coordinate to P_B(Yeates et al. 1988), changes in (M)214 may very well contribute to changes in P_{A,B} Q_y absorbance through minor variations of the P_B-(M)H202 bond.

3.9.2 X-ray crystallography and anomalous scattering of Zn- and Zn-β-RCs. To

elucidate the type, coordination state and occupancy of the bacteriochlorin cofactors in Zn-BChl-containing RCs, the crystal structures of the Zn-RC and Zn- β -RC were solved by X-ray diffraction to a resolution of 2.85 Å. The refinement statistics are listed in Appendix 2. Overall, the structures resemble that of the native RC (PDB entry 2J8C). In both the Zn-RC and Zn- β -RC structures, the iron, carotenoid and all the bacteriochlorin cofactors are present.

An electron density map was computed from the anomalous difference data collected above the Zn-edge of the two RC structures to reveal the location of Zn^{2+} ions within the two structures (Table 3.3 (Bearden 1967)). A peak greater than 4σ was observed in the

anomalous maps at the center of all bacteriochlorin-type cofactors indicative of the presence of Zn^{2+} ions at these sites. At the wavelength used for data collection (1.12709 Å), the anomalous signal from Mg^{2+} is weak and would not significantly contribute to the density observed in the map. The signal from iron can be used as an internal standard, and indeed peaks at 8.7 σ and 10 σ were observed at the iron site in the Zn-RC and Zn- β -RC structures, respectively. In the Zn-RC structure, five of the bacteriochlorin cofactors appear to be bound at full occupancy based on refined Zn^{2+} ion B-factors of 58 Å² or less and anomalous map peaks >12 σ . In contrast, the larger B-factor of the Zn atom at H_B site (86 Å²), in combination with the lower anomalous map peak (7σ) , imply a lower occupancy and/or a higher degree of disorder of this cofactor. The H_B cofactor in the Zn- β -RC structure exhibits even lower occupancy and/or greater disorder (Table 3.3). These values for the H_B cofactor in both the Zn-RC and Zn- β -RC, relative to the values for the other bacterochlorin cofactors, indicate that Zn-BChl is not well-occupied in the H_B pocket, and perhaps less well-occupied at this site in the $Zn-\beta-RC$ than in the Zn-RC. This decrease in occupancy may provide a rationale for the absence of a Q_x transition H_B peak (Figure 3.17 (Neupane et al. 2012)), by showing that there is some degree of structural perturbation present at the H_B site of the Zn-RC and Zn- β -RC. Interestingly, the B-factors of the amino acid residues surrounding the H_B cofactor were similar to those in the rest of the protein, indicating that the observed disorder was centered on the Zn-BChl molecule itself, and not the surrounding protein.

Table 3.3. Anomalous map statistics for metals in the Zn-RC and Zn- β -RC from x-ray

Site	Zn-RC				Zn-β RC			
	Omit	Anomalous	Zn B-	Leu/His-Zn	Omit	Anomalous	Zn B-factor	Leu/His-Zn
	Fo-Fc	map peak	factor	distance	Fo-Fc	map peak	(A^2)	distance (A)
	peak	(σ)	(Å ²)	(Å)	peak	(σ)		
	(σ)				(σ)			
\mathbf{P}_{A}	25	13	50	2.1	25	16	43	2.2
$\mathbf{P}_{\mathbf{B}}$	25	13	50	2.1	24	14	44	2.2
\mathbf{B}_{A}	25	14	44	2.2	25	15	39	2.3
B_{B}	24	14	39	2.4	25	16	37	2.5
H_{A}	20	12	58	3.2	26	15	43	2.3
H_{B}	13	7	86	3.6	11	4	113	3.9
Fe	24	9	45		24	10	41	

diffraction datasets.
In comparison to the H_B site, the B_B, P_{A/B}, and A-branch Zn-BChls of the Zn- β -RC and Zn-RC appear to be relatively well-ordered and fully occupied, based on the anomalous signal intensity and B-factor values, which are similar to the mean values of each respective structure (Table 3.3). A side by side comparison of the H_A cofactors of the Zn-RC and Zn- β -RC is shown in Figure 3.19. For Zn-BChl in sites with a His residue available for coordination, the imidazole ring is observed 2.1 to 2.4 Å from the Zn²⁺ ion. At the Zn-RC H_A site (with a Leu present), the side chain is separated by 3.2 Å from the Zn²⁺ ion of the Zn-BChl. Although at 2.85 Å resolution for these structures the error estimate of these distances is ±0.3 Å, at all of the Zn-BChls the electron density maps indicate the absence of an externally-derived metal ligand, such as water. Therefore, I suggest that the major structural difference between the A-branch electron transfer carriers in the Zn-RC and Zn- β -RC is the coordination state of the Zn²⁺ ion in the H_A Zn-BChl, which is 4-coordinate in the Zn-RC and 5-coordinate in the Zn-RC.



Figure 3.19. Stick models and electron density (mesh) of the HA cofactor and axial (M)214 residues from the crystal structures of the (A) Zn-RC and (B) Zn- β -RC. The distances from the axial residues to the macrocycle centers are 2.1-2.4 Å and 3.9 Å for the Zn-RC and Zn- β -RC, respectively. Color code: teal, carbon atoms; blue, nitrogen atoms; red, oxygen atoms. Figure reprinted with permission from Saer et al. (Saer et al. 2013b).

3.9.3 Time-resolved spectroscopy of wild type, (**M**)**L214H**, **Zn-, and Zn-\beta-RCs.** The fit in the Q_y region for the decay of P*, representative of forward electron transfer to the nearby B_A cofactor (and subsequently to H_A), yielded time constants of 3.8 ps for the wild type RC, 3.9 ps for the Zn-RC, 4.8 ps for the (M)L214H RC, and 5.5 ps for Zn- β -RC (Table 3). Thus, initial electron transfer takes place on the picosecond time scale for all four RCs, although the rates of the wild type RC and Zn-RC group together, separately from the rates of the (M)L214H RC and Zn- β -RC. A table of all the time constants and charge separation yields for the RCs used in this study is presented in Table 3.4.

Sample	P* (fast)	$P^+I_A^-$	$ au_{IQ}{}^a$	$\tau_{rec}{}^{b}$	$\phi^{c} (P^{+}Q_{A})$
Wild type	$3.8\pm0.2\ ps$	$210 \pm 30 \text{ ps}$	0.2 ns	20 ns ^d	100 %
(M)L214H	$4.8\pm0.5\ ps$	$560\pm100\ ps$	$4.0\pm0.7\ ns$	$0.65\pm0.1~\text{ns}$	14 %
Zn-RC	$3.9\pm0.7\ ps$	$290\pm50\ ps$	$0.4\pm0.1\ ns$	$1.1 \pm 0.2 \text{ ns}$	74 %
Zn-β-RC	$5.5\pm0.2\ ps$	$640 \pm 90 \text{ ps}$	$6.4\pm0.9ns$	$0.71 \pm 0.1 \text{ ns}$	10 %

 Table 3.4. Calculated transient state lifetimes, reaction rates and final yields of charge separation for RCs in this study based on global analysis.

^aCalculated rate of forward electron transfer from I_{A}^{-} to Q_{A} using Equation 3 in Section 4.6 .

^bCalculated rate of recombination using Equation 2 in Section 4.6.

 $^{c}Calculated yield of P^{+}Q_{A}^{-}$ based on the amount of P^+ bleaching amplitude (measured at

860 nm) at the end of the measurement window compared to 45 ps post excitation.

^dBased on measurements in which Q_A was removed from the RC.

Transient absorption difference spectra from 500 to 725 nm for all four RCs at 25 ps after excitation are shown in Figure 3.20. The spectra were normalized to the P* bleaching maximum near 600 nm at 0.5 ps. In the wild type RC, this spectrum is representative of the $P^+H_A^-$ state, however, as the other RCs in this study may exhibit different electron transfer kinetics, the spectrum at 25 ps may include features of other RC intermediate states, and thus this state is denoted P^+I_A in the mutants (i.e. I_A denotes a combination of H_A and B_A anions). In the Zn-RC, there is an absorption decrease near 560 nm, attributed to bleaching of the Zn-BChl in the H_A site because this wavelength corresponds to the Q_x absorption maximum of this cofactor in the ground state (Figure 3.18). Like the ground state transition, the magnitude of this bleaching is substantially less than that of the corresponding H_A bleaching in the wild type RC at ~545 nm. The difference in Q_x transition extinction coefficients between the wild type and Zn-RC may in part account for this amplitude difference, however the exact reason remains unclear. In contrast, the magnitude of absorbance decrease near 600 nm, which represents ground state bleaching of P on this time scale, is nearly identical in the wild type RC and Zn-RC.



Figure 3.20. Transient absorption difference spectra of the Qx transition of wild type (WT) (M)L214H, Zn-, and Zn- β -RCs 25 ps after excitation, representative of the P⁺I_A⁻ state. The data were normalized to the Q_x bleaching maximum of P* (~ 600 nm) at 0.5 ps post-excitation. Figure reprinted with permission from Saer et al. (Saer et al. 2013b).

In the Zn- β -RC there is no ground state absorbance in the 545 to 560 nm region (Figure 3.18), and therefore no ground state bleaching in this region of the difference spectrum (Figure 3.18). However the bleaching at ~600 nm is increased compared to both the wild type RC and Zn-RC. This is consistent with ground state absorption measurements of the Zn- β -RC at low temperatures, which show that the H_A Zn-BChl absorbs maximally at 598 nm, overlapping the ground state bands of P, B_A and B_B (Figure 3.18 (Neupane et al. 2012)). The red shift from ~545 to ~600 nm in both the ground state absorption spectrum and the difference spectrum of the (M)L214H RC results from conversion of BPhe to Hiscoordinated BChl, whereas in the Zn- β -RC the analogous shift is consistent with a Zn-BChl coordinate change at H_A from 4- to 5-coordinate.

A prominent feature of the P⁺I_A⁻ absorption spectrum of all these RCs is the large broadband absorption increase in the ~620 to 725 nm region (Figure 3.20), primarily due to anion absorption in this region. The peak of this absorbance increase shifts from ~660 nm in the wild type RC to ~680 nm in the (M)L214H RC, as shown previously (Kirmaier et al. 1995a). In both the Zn-RC and Zn- β -RC, the portion of this maximum is intermediate in wavelength between that of the wild type RC and the (M)L214H RC. The fact that the two Zn-BChl-containing RCs share a P⁺I_A⁻ absorption maximum at a similar wavelength, and which differs from that of both the wild type RC and the (M)L214H RC indicates that the position of the anion absorption peak is sensitive to both the presence and identity of the metal associated with the H_A bacteriochlorin, but insensitive to its coordination state.

The absorbance between ~620 and 725 nm associated with the I_A^- anion decays in the wild type RC with a time constant of 200 to 250 ps, as shown for the 675 nm wavelength in Figure 3.21, in agreement with previous work (Lin et al. 1996; Lin et al. 2009; Kirmaier et al.

1995b; Martin et al. 1986). The decay of the I_A^- anion band in the Zn-RC has a time constant of ~290 ps (Fig. 3.20, Table 3.4), as seen previously (Lin et al. 2009). In both the (M)L214H mutant and Zn- β RCs, much longer time constants of ~560 ps and ~640 ps, respectively, were resolved from the fitting (Table 3.4). Note that minima of larger uncertainties (± 50 ps) were found in the fits for the (M)L214H RC and Zn- β -RC compared to the wild type and Zn-RCs. The analysis of this complication will be given in the Discussion section.



Figure 3.21. Kinetics of $P^+I_A^-$ state decay at 675 nm for wild type (WT), (M)L214H, Zn-, and Zn- β -RCs measured up to 5500 ps. Spectra were normalized to the maximum amplitude increase at 675 nm. Open circles represent raw data points, and solid lines are representative of fits to the data. Figure reprinted with permission from Saer et al. (Saer et al. 2013b)

In Figure 3.22, the fitting results for each type of RC are represented as EADS (van Stokkum et al. 2004). Once again, as in section 3.6, this representation allows simplified visualization of the spectral progression associated with the decay times of the fit, and is not meant to depict the spectra of the actual excited and charge separated state populations (which would require the application of a specific kinetic model). As can be seen, the values of the time constants for the hundreds of picoseconds EADS for the wild type RC and Zn-RC (210 ps and 290 ps, respectively) are more similar to each other than to the time constants found for the (M)L214H RC and the Zn- β -RC (560 ps and 640 ps, respectively). Furthermore, the EADS with the longest time constant in both the wild type RC and Zn-RC was non-decaying (*i.e.*, greater than 3 times the time window of measurement), whereas the (M)L214H RC and the $Zn-\beta$ -RC did not contain such a long-lived component. Thus, the kinetic behavior of the 4-coordinate Zn-BChl at H_A is more like that of a BPhe than a 5-coordinate (Zn-)BChl. All of the mutants, including the Zn-RC, however, appear to have lower $P^+Q_A^-$ yields than the wild type RC on the nanosecond time scale. This can be seen in Figure 3.22 by comparing the bleaching of P near 860 nm in the green EADS component that has a lifetime of hundreds of picoseconds, with that of the red (nanosecond, or non-decaying) component. In the wild type RC, there is no significant decrease in the amplitude of the bleaching signal over this time, as would be expected for 100% yield of charge separation. In contrast, each of the mutants shows a partial decrease in bleaching amplitude. In the case of the Zn-RC, this decrease in bleaching amplitude is relatively small (74% of the bleaching amplitude remains; Table 3.3), however this amplitude is still much larger than those of the (M)L214H RC and the $Zn-\beta$ -RC (14% and 10%, respectively; Table 3.3). In our experiments, the degree of P^+ remaining in the (M)L214H RC at the end of the measurement, as indicated by the difference in bleaching

amplitude at 970 nm between 45 ps post excitation and the end of the measurement window, was less than reported previously by Kirmaier *et al.*(Kirmaier et al. 1995b) because our experiments used a longer measurement window (5.5 ns vs. 2.7 ns), which revealed additional amounts of ground state recombination. Note that there is no evidence for any yield loss on the picosecond time scale in either the wild type or mutant RCs. This can be seen by the invariance between the blue (few picoseconds) and green (hundreds of picoseconds) EADS near 830 nm in Figure 3.22. At this wavelength, there is essentially no contribution in the picosecond spectrum from stimulated emission, and so one can monitor the decay of ground state bleaching independently.

In summary, the studies on Zn-BChl-containing RCs presented herein highlight their usefulness as an investigative tool in understanding the properties of the RC pigments. The above results show that the presence of a metal center in the H_A chlorin results in a shifting of the transient absorption spectrum associated with the $P_+I_A^-$ state. Furthermore, the presence of an H_A metal center is associated with RC ground state recombination within a 6 ns measurement window. Introducing a fifth coordinate to the H_A Zn-BChl via site-directed mutagenesis results in an RC in which ground state recombination appears to be the dominant fate of the P⁺H_A⁻ state. In addition to these kinetic differences, the ground state absorbance spectroscopy and x-ray crystallography data suggest that Zn-BChl-containing RC assemble with a partial chlorin occupancy of the H_B site.



Figure 3.22. Evolution associated difference spectra (EADS) of the Qx and Qy transitions of wild type (WT), (M)L214H, Zn-, and Zn- β -RCs. The solid black line is a zero absorbance reference line. Each RC was fit to a fast < 10 ps component, a hundreds of ps component, and a nanosecond or non-decaying component. Component lifetimes are representative of best fits to the transient absorption spectrum datasets using a least squares fitting approach. Deviations represent the overlap of 5% variations in the χ^2 (goodness of fit) values between independent fittings of the Q_x and Q_y datasets. Figure reprinted with permission from Saer et al. (Saer et al. 2013b).

4. DISCUSSION

4.1 Pigment discrimination at H_A. The foregoing low temperature spectroscopy data (Section 3.3) support a hypothesis that the presence of BChl or BPhe in the H_A pocket is determined solely by the presence or absence of an axial residue capable of Mg²⁺ coordination. Although it was plausible that the RC H_A pocket could de-chelate a BChl Mg²⁺ ion through steric exclusion via the bulky (M)L214 side chain, the absorbance spectra of RCs containing the (M)L214G/A substitutions, which are neither bulky nor able to coordinate a Mg²⁺ ion, resemble the wild type spectrum more closely than the β mutant spectrum. Furthermore the crystal structures showed that the bacteriochlorin composition in the P, B and H sites of the (M)L214G/A RCs is the same as in the wild type protein.

In the low temperature spectrum of the (M)L214G mutant (Figure 3.4), however, small decreases in BPhe absorbance are observed for both the Q_x and Q_y transitions. These data are in agreement with the extraction experiment, which showed a slight increase in the BChl/BPhe ratio for the (M)L214G RC compared to the wild type (~2.6 vs. ~2.0). Thus, it is plausible that some RCs in this population may lack a BPhe cofactor at H_A. The data disagree with the (M)L214G X-ray crystallographic dataset, however, because the B-factors associated with the atoms of the BPhe cofactor at H_A do not differ from the average of the RC. One possible explanation for this is that the (M)L214G crystal was enriching for RCs containing a BPhe molecule at H_A during formation. Experiments aimed at supplementing the (M)L214G RC with excess BPhe may shed light on this matter. In addition to a missing BPhe cofactor, it is possible that the Q_A cofactor was absent in (M)L214G RC samples because this RC's recombination kinetics were similar an RC which the Q_A quinone was removed. This possibility does not seem likely, however, because an incubation of the (M)L214G RC with excess ubiquinone 10 did not significantly change the light minus dark difference spectrum of this RC (Appendix 5).

Our results are complementary to and extend previous studies on "cavity mutants" of the *R. sphaeroides* RC, in which His axial ligands to the P BChls (at positions (M)202 and (L)173), were changed to Gly residues, and proposed to introduce a cavity in the vicinity of the P BChls. Although crystal structures were not presented, Stark spectroscopy studies and pigment analysis demonstrated that those RCs assemble with a native pigment composition (Goldsmith et al. 1996). Furthermore, water molecules were proposed to act as a fifth ligand to the P BChls in the cavity mutants, and these waters appeared to be switched to other coordinating small molecules by incubation of the RC in the appropriate solute (Goldsmith et al. 1996). The results with mutations at H_A enrich our understanding of the complexity in pigment discrimination within RCs, by indicating that not all binding pockets conform to a single set of rules when it comes to populating these cofactor binding sites. Although adventitious ligands such as water may compensate for the loss of an imidazole group in the P BChls, such polar ligands do not appear to be suitable as BChl Mg²⁺ ligands when the (M)214 Leu side chain is absent from the H_A pocket. This difference may stem from the fact that the H_A pocket is imbedded more deeply in the membrane bilayer than the P region, and hence less likely to incorporate water (Yeates et al. 1987). Instead, the void is occupied in part by an alternative conformation of the phytyl tail of B_A .

An analogous structural plasticity in the P region of the RC was found in "heterodimer" mutations in *R. capsulatus* in which one of the P BChls was changed to BPhe by substitution of Leu for a His ligand at position (M)200 (the analogous residue in *R*.

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sphaeroides is (M)202) of the RC (Bylina and Youvan 1988; Kirmaier et al. 1988). In an *R. sphaeroides* (M)L214H/(M)H202L double mutant, however, substitution of Leu for a His ligand at this/these position(s) failed to yield a heterodimer, and the resultant RC incorporated two BChls at P for unknown reasons (Heller et al. 1995). Those results demonstrate that even when the coordinating axial (M)H202 residue is substituted with a Leu residue, the P binding pocket is capable of incorporating BChl. Although it is conceivable that an adventitious ligand was incorporated into the binding pocket of the P BChls (in the (M)H202/(M)L214H double mutant), those results raise additional questions about the nature of pigment selectivity in RCs as well as in the biogenesis of (B)Phes.

With respect to pigment selectivity, it would be interesting to investigate how pigment incorporation into the RC is affected by changes in the binding pockets of nearby cofactors, as was demonstrated by Heller *et al.* (Heller et al. 1995). One possibility is that, because residues (M)202 and (M)214 lie on the same helix, (Yeates et al. 1988) a perturbation of the protein backbone resulting from the combination of the (M)H202/(M)L214H changes may allow for the incorporation of adventitious ligands into the P pocket. The fact that the RC can incorporate BChl in the presence of imidazole also raises the question: how does the presence of these ligands prevent the demetallation of BChl in BPhe binding pockets?

Although the mechanisms underlying BPhe production are unknown, several groups have investigated the production of pheophytin (Phe) from Chl molecules in cyanobacteria and higher plants. The biological production of a Phe from a chlorophyll (Chl) is best understood in senescent plant materials. Shioi and coworkers partially purified a heat-stable "magnesium de-chelating substance", that catalyzed the conversion of chlorophyllide to pheophorbide (Shioi et al. 1996). The name of this compound was then changed to "metalchelating substance", because it was found that this substance was not specific for Mg^{2+} ions, and was able to chelate other divalent cations (Suzuki and Shioi 1999; Kunieda et al. 2005). Although this as yet poorly defined low molecular weight substance was implicated as functioning during leaf senescence, and therefore Chl degradation, it cannot be excluded that such a substance could function during Chl synthesis, to yield Phe for incorporation into RCs.

Other work on plant material, in this case on the source of Phe in the photosystem II RC (Ignatov and Litvin 1994), pointed to a pre-Chl origin of Phe in which a branch-point in the Chl biosynthetic pathway was proposed as a possible source of Phe in etiolated leaves. The authors suggested that the magnesium chelatase enzyme itself was responsible for Mg²⁺ removal (Ignatov and Litvin 1994). Such a branch-point has not been reported in the BChl biosynthesis pathway of purple phototrophic bacteria despite extensive mutagenic analyses (Willows and Kriegel 2009). Although it is possible that a dedicated de-chelating enzyme exists for the synthesis of RC (B)Phe, a mutant strain deficient in this activity has not been discovered thus far. Moreover, (B)Phe is functionally present only in RCs, and free (B)Phe is not found in non-senescent photosynthetic organisms. Alternatively, it is possible that the local environment of the (B)Phe-binding pockets within RCs, in general, results in the loss of Mg²⁺ from (B)Chl unless a coordinating ligand specific for the central metal is present. Because of the tendency for this metal ion to exist only in the penta- or hexacoordinated state in BChl (Callahan and Cotton 1987; Goldsmith et al. 1996; Evans and Katz 1975), it is conceivable that the Mg^{2+} is removed from the macrocycle in the H_A site in part because BChl is simply not stable in the tetracoordinate state (Evans and Katz 1975; Katz 1968).

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The issue about BPhe's origin was further explored in my thesis work through experiments involving biochemical pigment substitution in purified RCs. One interesting result derived from the substitution experiments was the capability of the (M)L214G mutant RC to seemingly incorporate metal-containing chlorins. Although it is not entirely clear, the fact that the absorption spectrum of the (M)L214G RC, but not the wild type RC, changed upon substitution with both Chl and BChl supports this conclusion (Figure 3.15). When incubated in a molar excess of BChl a, the (M)L214G RC exhibits a small absorbance decrease at ~765 nm with a concomitant increase in ~775 nm absorbance. Furthermore, the (M)L214G RC demonstrated a large absorbance decrease at ~765 nm when incubated with a molar excess of Chl a. Based on these findings, it appears that a bulky residue at (M)214 contributes to the selectivity (at least *in vitro*) of BPhe over BChl in the H_A pocket when no axial ligand to the chlorin is provided. Because the (M)L214G mutant RC assembles exclusively with BPhe at H_A *in vivo*, it appears that axial ligation of Mg²⁺ is the most important determinant for BChl incorporation.

4.2 Spectroscopic and structural consequences of nonpolar (M)L214 mutations.

An interesting consequence of substituting (M)L214 with small-volume, nonpolar side chain residues in the *R. sphaeroides* RC is the shifting of the Q_x transition corresponding to the H_A BPhe to higher energy. It was previously suggested by Bylina *et al.* that an analogous blueshifting may be attributed to changes in the hydrogen bonding interaction between a carbonyl group on the H_A BPhe macrocycle and a nearby Glu at position (L)104 (Bylina et al. 1988; Yeates et al. 1988). Specifically, when (L)E104 was changed to a weaker proton donor (Gln), or to a Leu incapable of hydrogen-bonding to the BPhe, the Q_x absorbance maximum of H_A became more similar to that of H_B (a shifting to higher energy was found). In the case of a RC (L)E104L mutant, the Q_x transitions of H_A and H_B were almost overlapping. A blue shift similar to what was reported for the (L)E104Q mutant was observed in my (M)L214G and (M)L214A mutants (Figure 3.4B), but the crystal structures show that these shifts are independent of changes in macrocycle hydrogen-bonding interactions. I speculate that the changes in the Q_x transition of H_A observed in the (M)L214G and (M)L214A mutants are induced by the disorder in the B_A phytyl tail, related changes in the binding of detergent, and possibly disordered water molecules.

The X-ray structures of the (M)L214G/A mutants reveal that this residue affects the orientation of the B_A phytyl tail (Figure 3.9), and offer an explanation for the changes in the low temperature absorbance spectra of the RCs with these low-volume side chains. Although the influence of the phytyl tail in the direct tuning of the electrochemical midpoint potential of a chlorin is not well understood and considered to be negligible, the tail was thought to affect the packing of protein residues and nearby chlorins (Scheer 2006). Our crystal structures indicate that, other than the substituted side chain itself, there is not a direct effect of the (M)214 side chain substitution on the electronic properties of H_A (*i.e.*, there appear to be no changes in the distance between the H_A macrocycle and protein atoms that would give rise to changes in the electronic properties of the H_A BPhe). Therefore, the absorbance changes in the Q_x and Q_y transitions of H_A appear to stem predominantly from changes in the B_A phytyl tail and perhaps the presence of disordered water, as an indirect effect of the change of (M)L214 to smaller, nonpolar moieties. These mutational changes are also accompanied by a broadening of the Q_y absorbance band of B_A in the (M)L214G mutant (Figure 3.4A), perhaps reflecting a population of mutant RCs that assume a range of alternative B_A phytyl tail configurations, as indicated by the crystal structure.

Although the X-ray diffraction data from (M)L214A RC crystals indicate that some degree of B_A phytyl tail disorder is present (Figure 3.9C), this disorder appears to be less significant than in the (M)L214G RC crystal, because electron density for the "right" conformation is continuous whereas electron density for the alternative conformation is weaker. It could be argued that this difference in electron density arises solely from the lower resolution of the (M)L214A data relative to the (M)L214G structure, but in the absorbance spectra the broadening of the BChl $B_A Q_y$ band is seen most prominently in the (M)L214G mutant (Figure 3.4A). Although to a lesser extent, the BChl $B_A Q_y$ band is also broadened in the (M)L214A RC without a corresponding increase in the amplitude of the band (Figure 3.5). In combination with the similarities between the (M)L214A and (M)L214G Q_x transitions, these data indicate some disorder or motion in the (M)L214A B_A phytyl tail, although it appears to be insufficient to allow the adoption of alternative conformations to the same degree as in the (M)L214G mutant.

The RC contains the electronically active cofactors within well-insulated tunneling pathways that prevent molecules such as intramembrane quinones from interacting with RC pigments and thus potentially affecting rates of electron transfer. *In vivo*, the RC is surrounded by light-harvesting complex 1 (LH1), which may act as a barrier between the quinone pool and the RC (Bahatyrova et al. 2004; Lilburn et al. 1995; Qian et al. 2008). However, it was shown that 25 to 30% of the native quinone pool is retained in an isolated RC/LH1/PufX preparation (Comayras et al. 2005), evidently because quinones bind to these proteins and may be located within the space between the RC and LH1. In fact, a recent report on the structure of the RC/LH1/PufX core complex revealed that multiple quinones could be accommodated in this space (Qian et al. 2013). In addition to the insulating shield

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provided by the RC protein matrix, the long, aliphatic phytyl tails esterified to the BChl and BPhe macrocycles may also serve a similar role, at least *in vitro*. This possibility becomes apparent when the RC crystal structure is viewed parallel to the plane of the membrane, along the side of the A branch, which reveals a cofactor-filled hole (Figure 4.1A). From this perspective, the phytyl tails of the BChls (yellow, green) and BPhes (cyan), as well as the isoprenoid tail of the Q_A ubiquinone (blue), appear to insulate the macrocycles of B_A and H_A from the external milieu (Figure 4.1B). However, my low light intensity phototrophic growth experiments indicated that B_A tail conformation heterogeneity does not have a great effect on the *in vivo* efficiency of RC function, because the (M)L214G/A/C mutants grew as well as the wild type control strain (Section 3.4). Although it is plausible that these cofactor hydrophobic tails spontaneously adopt alternative configurations, there has been little account of the mechanisms by which these tails assume their native positions, or what factors might govern their 3-dimensional configurations. However the *R. sphaeroides* B_A phytyl tail "right" configuration is conserved amongst the other three type 2 RC crystal structures (Figure 4.2).

The *in vivo* efficient functionality of *R. sphaeroides* RCs containing these structural perturbations (*i.e.*, the wild type-like phototrophic growth of mutants under extremely low intensity illumination) testifies to the robustness of this protein in the catalysis of light-driven excitation energy transduction. However, kinetic analysis of electron transfer reactions in purified RCs reveals that the (M)L214G RC is significantly impaired in electron transfer from H_A to Q_A , as is the (M)L214A RC to a lesser extent (Pan et al. 2013).



Figure 4.1. Organization of the pigment cofactors in crystal structures of the RC. (A) Overview of the wild type RC (PDB 2J8C) with the region of interest enclosed by the green circle. (B) Close-up of the area indicated by the green circle in Panel A; the phytyl and isoprenoid tails of the BChls, BPhe and quinone on the A branch of the RC insulate electronically active pigment macrocycles by acting as a barrier against the external environment. Colour code: Green, P_A tail; yellow, B_A tail; orange, B_A macrocycle; cyan, H_A tail; magenta, H_A macrocycle; blue, Q_A tail; gray, protein. (C) View of the equivalent region highlighted in Panel B, but now in the (M)L214G mutant, with the B_A phytyl tail modeled in the "left" orientation. The ring 1 acetyl group of the H_A macrocycle (magenta) and ring 4 of the B_A macrocycle (orange) are exposed to the RC-external milieu. Figure modified with permission from Saer et al. Copyright 2013 American Chemical Society. (Saer et al. 2013a).



Figure 4.2. Orientation of the BA phytyl tails in RCs for which high-resolution data are obtainable. **A**, *R. sphaeroides* (PDB 2UWV). **B**, *Blastochloris viridis* (PDB 3T6D). **C**, *Thermochromatium tepidum* (PDB 1EYS). **D**, Cyanobacterial Photosystem II (PDB 3BZ1). **E**, the (M)L214G RC. Figure reprinted with permission from Saer et al. Copyright 2013 Amercian Chemical Society. (Saer et al. 2013a).

In contrast to the (M)L214G RC, the (M)L214C, (M)L214V and (M)L214I RCs all exhibited red shifts of the $H_A Q_y$ transition (Figure 3.4A). It appears that the presence of a bulky moiety at the γ position of (M)214 (either SH in the case of Cys, or two methyl groups in the case of Val and Ile) is sufficient to cause a detectable shift. Replacement of (M)L214 with Met has the opposite effect despite presenting a bulky moiety predicted to be at the same position as Leu side chain in the wild type RC. It is possible in the case of the (M)L214M mutant that the blue-shift of the $H_A Q_y$ transition stems from the establishment of hydrogen bonds between the Met residue SD atom and the pyrrole ring nitrogen protons of the BPhe macrocycle. X-ray crystallography of the (M)L214M RC may shed more light on whether the Met residue and the BPhe are able to hydrogen bond.

4.3 Spectroscopic and structural consequences of polar (M)L214 mutations. The

mutant RCs containing Asn and Gln amide side chains at (M)214 incorporated BChl into the H_A pocket. These proteins resemble the (M)L214H (β -type) mutant RC originally described by Kirmaier *et al.* (Heller et al. 1995; Kirmaier et al. 1991). In terms of *in vivo* activity, strains harboring these RCs exhibited similarly impaired phototrophic growth kinetics under a low light intensity (Figure 3.8). However, the absorbance band shifts induced by these three β -type mutations were very different from each other (Figure 3.6). Because the Q_y bandhifts of the (M)L214N and (M)L214Q RCs differed from each other more than from the (M)L214H RC (Figure 3.6A), I suggest that the absorbance spectra of H_A BChls are not greatly affected by whether the Mg²⁺-ligating atom is an imidazole nitrogen or an amide oxygen. In these cases, the possibility that the amide nitrogen atom acts as a ligand is excluded due to the participation of the nitrogen's lone electron pair in a resonance structure of the amide group, the location of the ND1 atom of (M)N214 within H-bonding distance of

the main chain carbonyl of (M)Y210, and because of the need for loss of a proton from the amide group to form a Mg^{2+} -N ligand.

The side chain of the residue replacing the Leu in the (M)L214N, (M)L214Q, and (M)L214H RCs differs in length and volume. The Asn side chain is shorter than the Gln side chain, but it offers equivalent atoms to coordinate the Mg²⁺ ion. The (M)L214N and (M)L214Q RC H_A Q_y absorbance bands differed by about 25 nm (Figure 3.6A), and so the length and/or volume of the Mg²⁺-coordinating side chain appears to have a major effect on the energy of the $H_A Q_y$ absorbance band, with the longer Gln side chain resulting in a higher energy H_A band in the Q_y region. Indeed, studies of BChls in a variety of organic solvents demonstrated that the Q_v transition is sensitive to the nature of the axial ligand to the pigment. Specifically, the Q_y transition shifts to a higher energy when the Mg²⁺ ion approaches the plane of the macrocycle due to a stronger metal-macrocycle interaction (this interaction being strongest in an in-plane Mg²⁺, as is the case in hexacoordinated BChls), and vice versa (Fiedor et al. 2008; Hanson 1988). The distance between the H_A Mg²⁺coordinating atom in the (M)L214H side chain and the BChl Mg²⁺ may be intermediate between the distances in the (M)L214N and (M)L214Q RCs; if so, this difference would account for the intermediate position of the $H_A Q_y$ absorbance band relative the $H_A Q_y$ bands of the (M)L214N and (M)L214Q RCs, but the coordinates of the (M)L214H mutant RC are unavailable. The atomic structures of Gln and His show that the Mg-ligating atom and the α carbon of the amino acid are separated by an equal number of bonds, thus a structural dataset of the (M)214H mutant is a must before an accurate comparison can be made.

Based on the available data, I suggest that the distance between the metalcoordinating atom of the RC protein and the Mg^{2+} ion of (B)Chls, and therefore the position of the Mg^{2+} relative to the plane of the (B)Chl macrocycle, is the major determinant of the Q_y band energy in absorbance spectra, rather than the identity of the coordinating atom (*i.e.*, a His nitrogen atom or an oxygen atom from Asn or Gln). Greater distances of coordinating atoms from the Mg^{2+} , as is the case with Asn, result in a Q_y absorbance maximum at a longer wavelength, whereas lesser distances (as with His or Glu) result in correspondingly shorter wavelength of the Q_y absorbance peaks.

4.4 Effects of changing the volume of (M)214 on the structure of RCs in vivo. The

fact that the pigment composition of the (M)L214G RC is the same as the wild type, in addition to its similar rate of photoheterotrophic growth, led me to investigate the question of whether other phototrophs naturally exist with small, nonpolar moieties axial to BPhe molecules in their RC. One hundred different organisms were chosen by using the *R*. *sphaeroides pufM* gene in a BLAST search (excluding *R. sphaeroides* from the results). From there, a multiple sequence alignment (using Clustal Omega) of the *pufM* gene from the 100 sequenced organisms was performed (Sievers et al. 2011; Goujon et al. 2010). While not completely conserved among the 100 predicted *pufM* genes, all but five organisms contained a Leu residue at the position analogous to (M)214 in *R. sphaeroides*. The five exceptions, Met or Val, are listed in Table 4.1 below.

Table 4.1 Organisms that do not encode a Leu at the analogous (M)214 position from a

Organism Name	NCBI Accession	Residue at position		
	No.	analogous to (M)214		
Rhodobacter veldkampii DSM 11550	BAC54030_1	Methionine		
Erythrobacter sp. NAP1	WP_007164992_1	Valine		
Porphyrobacter sp. AAP82	WP_017664717_1	Valine		
Rhodovulum sp. PH10	WP_008383958_1	Valine		
Lamprocystis purpurea	WP_020503997_1	Valine		

search of 100 genome s	equences that contain	a <i>pufM</i> homologue
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The sequence alignment of 100 representative organisms (Appendix 3) showed that there is a strong conservation of a Leu residue at a position that would be axial to the photoactive BPhe in the RC of various photosynthetic bacteria. When this residue is not Leu, another bulky hydrophobic residue takes its place (*i.e.*, Met or Val). One reason for incorporating a bulky residue at (M)214 could be explained in part by my results in the adventitious ligand experiments on cells grown in the presence of imidazole. In the (M)L214G mutant, purification of the RC in the presence of imidazole resulted in the apparent loss of one of the cofactors, whereas the wild type RC appeared resistant to this effect. Thus, the bulky (M)214 residue appeared to provide a means by which RCs can properly assemble in the presence of solutes such as imidazole, which otherwise may act as adventitious ligands to BChl. Although it is unlikely that these solutes are present in high concentrations in laboratory growth media (unless deliberately added), one cannot exclude the presence of such substances in the habitats in which these bacteria live and grow. High-throughput analyses (*i.e.*, mass spectrometry) of the prevalence of different solutes found in common habitats of purple bacteria could provide a clearer picture on this matter.

4.5 *In vitro* **performance of RCs with nonpolar moieties at H_A.** The results described in section 3.6 of this thesis demonstrate that the (M)214 residue plays a role in modulating the rate of electron transfer through the H_A cofactor. In summary, the primary events of charge separation leading to the formation of the P⁺H_A⁻ (or P⁺I_A⁻) state are not significantly altered in any of the mutants. This is in contrast to the (M)L214H mutant, in which the formation of P^{*} \rightarrow P⁺I_A⁻ takes approximately twice as long as the WT RC (~ 6 ps in the (M)L214H mutant (Kirmaier et al. 1995b)). Thus, the formation of P⁺H_A⁻ appears to be affected primarily by the presence or absence of a pentacoordinate metal center in the H_A cofactor. The fate of the P⁺H_A⁻ state, however, appears to be highly dependent on the volume of the (M)214 side chain. The (M)L214M mutant, with a side chain similar in bulkiness to the wild type Leu residue, exhibits efficient H_A to Q_A electron transfer dynamics, whereas the other mutant RCs with smaller side chains at (M)214 do not. In fact, the (M)L214G/A/C mutants appear to contain traces of the P⁺H_A⁻ state even ~6 ns after the excitation flash. This long-lived state is associated with recombination of P⁺H_A⁻ to the ground state, resulting in a partial loss of charge separation among a given population of RCs, as indicated by the data in Figures 3.13 and 3.14, where recovery of P band bleaching is evident in mutants with longlived 675 nm P⁺H_A⁻ transients.

A fitting of the time-resolved spectral datasets was performed as outlined in Pan et al (Pan et al. 2013). These data were fitted to a set of exponential decays using a sequential model (*i.e.*, $A \rightarrow B \rightarrow C$). The resultant EADS obtained from these fits, in addition to a set of proposed models to explain the kinetic complexities (explained further in the following paragraph), are shown in Figure 4.3.



Figure 4.3. Evolution associated difference spectra (EADS) and kinetic models of the wild type (WT) and (M)L214G/A/C RCs. Middle panels: EADS obtained from global fitting analysis of time-resolved spectral datasets for the WT and (M)L214G/A/C RCs. Panels on the left column represent reaction schemes of the WT and mutant RCs based on a static heterogeneity model. Panels on the right column represent reaction schemes based on a dynamic heterogeneity model. Solid black arrows represent dominant reaction pathways, whereas solid gray arrows represent pathways that are unlikely to be a dominant one. Wavy arrows indicate $P^+H_A^-$ relaxation. Orange numbers represent time constants from the fits, as well as the calculated yields for each measured RC. Black numbers in the reaction schemes represent recombination rates obtained from the literature. Figure reprinted with permission from Pan et al. Copyright 2013 Amercian Chemical Society. (Pan et al. 2013).

The fitting results revealed the presence of extra decay components in the

(M)L214G/A/C mutant RCs compared to the wild type RC. In all of the RCs studied, all of three components were required to explain the fits to the experimental data during the initial phase of charge separation (*i.e.*, $P^* \rightarrow P^+B_A^- \rightarrow P^+H_A^-$). As mentioned in the Introduction (Section 1.4), fast components of 2.8 to 3.5, and 0.9 ps were resolved in all the RCs. These two components correspond to an electron transfer from P^* to B_A , and subsequently from $B_A^$ to H_A , generating the P⁺H_A⁻ state. The third component, with a lifetime of 10 to 12 ps, arises from a slower decay phase of the P* state, as has been discovered in RCs of R. sphaeroides, *R. capsulatus* as well as *Blastochloris viridis* (Holzwarth and Muller 1996; Huppman et al. 2002; Jia et al. 1993; van Stokkum et al. 1997; Lin et al. 1996). The nature of this slower decay phase has been previously suggested to be charge separation occurring from a more vibrationally relaxed state of P*, however, this proposal has never been examined in closer detail (Lin et al. 1996). As for the two faster phases, all the (M)L214 mutant RCs show evidence of this decay phase with a similar lifetime. In addition to these three components, all RCs exhibit a slower, hundreds of picoseconds decay component which is characteristic of electron transfer from H_A^- to either Q_A , or back to P^+ (recombination; Figure 4.3e-h). This decay rate appears to be correlated with the volume of the side chain at (M)214, thus acting as a kinetic branching point at which the behavior of the (M)L214 mutants begins to differ. The lifetime of this rate increases as the volume of the (M)214 side chain decreases. The time constants of the hundreds of picoseconds components were calculated to be 220 ps (for the wild type RC), 250 ps ((M)L214C), 290 ps ((M)L214A), and 330 ps ((M)L214G). Furthermore, a fourth decay component in the nanosecond time domain was required to adequately fit the datasets for the (M)L214G/A/C mutants (Figure 4.3f-h). Values of 6 to 10

ns (for the (M)L214C RC), 6 to 8 ns ((M)L214A), and 4 to 6 ns ((M)L214G) were obtained for this component,. In the (M)L214C and (M)L214A RC, this component is characterized by a slower electron transfer event from H_{A}^{-} to Q_{A} , as the 675 nm transient signal decreased in amplitude with no appreciable decrease in P^+ bleaching amplitude. In the (M)L214G mutant, however, there appears to be some degree of recombination to the ground state within this time domain, suggesting that the recombination reaction occurs with a faster rate in this RC than the wild type, (M)L214A, or (M)L214C RC. Finally, all RCs exhibited a non-decaying component with a lifetime greater than 3 times the time window of the experiment. In the wild type RC, this component contains no trace of the H_A^- signal at 675 nm, however the P⁺ bleaching at 865 nm is still present, implying that this component is dominated by RCs in the $P^+Q_A^-$ state. In the (M)L214G/A/C RCs, the non-decaying component spectrum contains traces of the H_{A} anion signal, as well as decreases in 865 nm bleaching amplitude. The degree of recombination, as evidenced by the loss of negative amplitude at 865 nm, is once again correlated with the volume of the side chain at (M)214, with the (M)L214G mutant showing the most pronounced amplitude loss. Thus, RCs with smaller (M)214 side chains are more likely to exhibit $P^+H_A^- \rightarrow$ ground state recombination from a state of P^+H_A that is relatively long-lived compared to that of the wild type RC.

To calculate the population of $P^+H_A^-$ and $P^+Q_A^-$ in the EADS for the measured RCs, the spectra were fitted to a linear combination of the $P^+H_A^-$ and $P^+Q_A^-$ spectra from the wild type RC using the expression EADS = $h[P^+H_A^-] + q[P^+Q_A^-]$, where *h* and *q* are values that specify the amount of $P^+H_A^-$ and $P^+Q_A^-$ in the EADS, respectively (Pan et al. 2013). For this calculation, it was assumed that there was a negligible amount of $P^+Q_A^-$ present in the hundreds of picoseconds EADS. The fits of the hundreds of picoseconds EADS from the (M)L214G/A/C RCs to that of the wild type RC are shown in Figure 4.4.



Figure 4.4. Overlay of the hundreds of picoseconds EADS for the (M)L214G, (M)L214A, and (M)L214C RCs (black dotted lines) with the 220 ps EADS of the wild type RC (gray solid lines). Figure reprinted with permission from Pan et al. Copyright 2013 Amercian Chemical Society. (Pan et al. 2013).

Fitting the hundreds of picoseconds EADS from the (M)L214C and (M)L214A RCs to that of the wild type RC resulted in deviations (i.e. a difference in amplitude) of less than 5% from each other. Thus, it was assumed that these two RCs contain 100% $P^+H_A^-$ after the initial steps of ET. In the (M)L214G mutant, the deviation was approximately 85%. Because the hundreds of picoseconds EADS of the (M)L214G RC exhibits a larger bleaching amplitude at ~600 nm, the remaining 15% deviation was assigned to $P^+B_A^-$. Application of this approach to the nanosecond-scale EADS revealed that the three mutant RCs contained mixed population of $P^+H_A^-$ and $P^+Q_A^-$ (compared to a ~100% population of $P^+Q_A^-$ in the wild type RC). In the (M)L214G RC, it appears that there is some evidence of recombination to the ground state, because it was found that 70% of the population of RCs was in the $P^+H_A^$ state, and 10% were in the $P^+Q_A^-$ state. For the non-decaying EADS of the mutant RCs, the populations were fitted to a linear combination of the 220 ps and non-decaying EADS of the wild type RC. Note that the values of h and q do not necessarily add up to 1 if recombination of $P^+H_A^-$ to the ground state in a sufficiently large fraction of the RC population has occurred. The results of h and q for the hundreds of picoseconds, nanosecond, and non-decaying EADS are compiled in Table 4.2.

	Wild type		(M)L214G		(M)L214A		(M)L214C		
	$P^+H_A^-$	$P^+Q_A^-$	$P^{+}B_{A}^{-}$	$P^{+}H_{A}^{-}$	$P^{+}Q_{A}^{-}$	$P^+H_A^-$	$P^{+}Q_{A}^{-}$	$P^+H_A^-$	$P^{+}Q_{A}^{-}$
hundreds of	100	0	15	85	0	100	0	100	0
picoseconds									
nanosecond	NA*	NA*	5	68	10	79	19	48	52
non-decaying	0	100	0	40	10	36	24	14	62

Table 4.2. Populations (in %) of charge-separated states in (M)L214 mutant RCs derived from linear combinations of the wild type hundreds of picoseconds and non-decaying EADS.

* No nanosecond EADS was obtained for the WT RC.

From the population values obtained in Table 4.2, in addition to the X-ray crystallography results, two reaction schemes, shown in Figure 4.3, were devised to explain the kinetic heterogeneity present in the (M)L214 mutants. Because the kinetic differences between the mutant RCs begin to be manifested once the $P^+H_A^-$ state is reached, the two models are displayed as beginning from this state.

In a static model (Figure 4.3a-d), it is assumed that different populations of RCs in the same sample are behaving differently. For the (M)L214A and (M)L214C mutants, the application of this model would suggest that one population of RCs is behaving like the wild type RC with fast forward ET from H_A^- to Q_A . Two other populations are associated with a long-lived P⁺H_A⁻ state. One of these populations decays in 15 to 20 ns solely via recombination to the ground state, and is represented by the P⁺H_A⁻ contributions to the nondecaying EADS. A third population of RCs decays from the P⁺H_A⁻ state on the nanosecond time scale via two possible pathways. In one pathway, the RCs recombine to the ground state in 10 ns. In the other pathway P⁺H_A⁻ decays via a slow phase of forward ET, generating P⁺Q_A⁻. For the calculation of the time constants in Figure 4.3b-d, the time constant of the appropriate EADS obtained in the fitting was divided by the calculated population of P⁺H_A⁻ in the EADS. As an example, I explain in the following paragraph the calculation of the time constant for slow forward electron transfer in the (M)L214C mutant from P⁺H_A⁻, yielding P⁺Q_A⁻.

To determine the rate of slow forward electron transfer from $P^+H_A^-$ to Q_A for the (M)L214C mutant (with a time constant of 24 ns), the 6 to 10 ns time constant for the nanosecond EADS was divided by a fraction of the subpopulation of RCs undergoing the slow phase of H_A^- to Q_A electron transfer. When moving from the nanosecond to non-

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decaying EADS for the (M)L214C mutant, it is evident that 10% of the RCs in the entire measured sample have entered the $P^+Q_A^-$ state anew (Table 4.2). However, in this static model, 3 different populations of RC behave according to different kinetic parameters, and so we need to find the fraction of the subpopulation that this 10% of the entire sample represents. Because this subpopulation would be included in the fraction of RCs that are in the $P^+H_A^-$ state in the nanosecond EADS, but not in the non-decaying EADS, we can look for the $P^+H_A^-$ populations that differ between these two EADS (Table 4.2) to find the fraction of RCs that this subpopulation represents; namely, 48% - 14% = 34% (35% when rounded to the nearest 5%). Thus we have 6 to 10 ns divided by (10/35) yielding 21 to 35 ns (the 24 ns value in Figure 4.3b was a result of using the least squares fitting value, however error in the fit produces the 21 to 35 ns range of values). Similarly, for the fraction of this subpopulation that recombines to the ground state, we can calculate the time constant as 6 to 10 ns divided by (25 / 35) = 8 to 14 ns. These calculations demonstrate that: (i) the (M)L214C RC contains a higher fraction of proteins capable of fast forward electron transfer from H_A^- to Q_A when compared to the (M)L214A mutant; (ii) the (M)L214C mutant is associated with fewer RCs that recombine to the ground state via the slower (15 to 20 ns) decay phase of $P^+H_A^-$; and (iii) the (M)L214C mutant $P^+H_A^-$ is also more likely to decay via slow forward electron transfer to Q_A than the (M)L214A mutant among the population of RCs with P⁺H_A⁻ states that decay in the nanosecond time domain (note that in both the (M)L214C and (M)L214A RCs, ground state recombination is still the dominant decay pathway for this subpopulation).

Using the static model on the (M)L214G mutant results in a total of four different subpopulations exhibiting kinetic heterogeneity. Unlike the (M)L214C and (M)L214A mutants, this RC lacks the slow decay phase of $P^+H_A^-$ to $P^+Q_A^-$ (Figure 4.3h). In this RC, sub-

nanosecond electron transfer is the only means of achieving the $P^+Q_A^-$ state, and all other long-lived subpopulations of $P^+H_A^-$ return to the ground state. In addition, there appears to be a subpopulation of (M)L214G RCs that exhibit a relatively rapid rate of ground state recovery, as evidenced by the small decrease in P^+ bleaching amplitude in the nanosecond EADS. Because this RC is the only one of the tested mutants exhibiting $P^+B_A^-$ character in the hundreds of picoseconds EADS (Table 4.2), this subpopulation of RCs was assigned to those which decay to the ground state via $P^+B_A^-$.

A comparison of the kinetics of the three mutant RCs using the static model reveals certain key points with regard to the volume of the (M)214 side chain. Firstly, it appears that the rate of forward electron transfer from $P^+H_A^-$ to Q_A in the hundreds of picosecond domain decreases as (M)214 side chain volume increases. Secondly, substitution of (M)214 with small, nonpolar moieties (cysteine included) results in two subpopulations of RCs containing a nanosecond-long $P^+H_A^-$ state. One of these populations behaves similarly among all RCs, and presumably exhibits similar kinetics to Q_A-depleted RCs, in which the RC recombines to the ground state in ~15 to 20 ns. The second of these subpopulations decays mainly via recombination to the ground state, although it is also capable of forward electron transfer to Q_A in the nanosecond time domain if the (M)214 side chain is a methyl group (Ala) or larger, with larger side chains exhibiting a higher degree of forward electron transfer. Combining this model with the x-ray crystallography data of the (M)L214G and (M)L214A RCs, the data indicate that the structural disorder in the B_A phytyl tail of RC mutants with smaller (M)214 side chains gives rise to populations of RCs that cannot efficiently undergo H_{A} to Q_{A} electron transfer. Exactly how much of this kinetic complexity can be explained by the

static model, as well as the underlying mechanism by which structural differences in B_A give rise to this complexity, is not clear.

In Figure 4.3i-l, the (M)L214C/A/G RCs are modeled in terms of dynamic heterogeneity. In a dynamic model, the different kinetics are ascribed to a relaxation in the energy of the $P^+H_A^-$ charge-separated state on the hundreds of picoseconds to nanosecond time scale. The application of this model arises from previous transient absorbance studies which suggested that the speed of an electron transfer event in the RC is faster than the energetic relaxation of charge-separated intermediates (*i.e.*, the higher energies of the transient excited states and anionic radicals in the RC do not fully relax before electron transfer takes place (Jia et al. 1995; Lin et al. 1996)) The general assumption of this model is that the $P^+H_A^-$ initially formed from P^* (termed $(P^+H_A^-)_i$) can relax to form two different intermediate states in the mutant RCs: $(P^+H_A^-)_m$ and $(P^+H_A^-)_f$. In the wild type RC, only $(P^+H_A)_i$ is formed, and it undergoes fast forward electron transfer to Q_A. In the mutants, however, the rate of electron transfer to Q_A is slow enough that relaxation to $(P^+H_A^-)_m$ and $(P^+H_A)_f$ compete with forward electron transfer, generating significant populations of these more relaxed charge-separated states. Electron transfer from these relaxed charge-separated states is associated with both a slower rate of forward electron transfer to Q_A, as well as an increased rate of recombination to the ground state. In the dynamic model (Figure 4.3i-1), the yield losses for recombination from $(P^+H_A^-)_m$ and $(P^+H_A^-)_f$ are calculated by measuring the decrease in P⁺ bleaching amplitude when transitioning from the hundreds of picoseconds EADS to the nanosecond EADS (for $(P^+H_A)_m \rightarrow \text{ground state}$), and from the nanosecond to the non-decaying EADS (for $(P^+H_A^-)_f \rightarrow$ ground state). The different populations of mutant RCs assigned to the charge-separated states were estimated by calculating the amount of

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 $P^+H_A^-$ relative to $P^+Q_A^-$ for each EADS in the same fashion as in the static model. According to the dynamic model, the substitution of (M)L214 with smaller, nonpolar moieties results in slower $H_A^- \rightarrow QA$ electron transfer, with smaller volume side chains associated with slower rates of forward electron transfer. These slowed electron transfer rates allow $P^+H_A^-$ to relax to lower energy states, resulting in recombination to the ground state, as well a relatively slow phase of forward electron transfer from H_A^- to Q_A .

The two models summarized above are useful in accounting for the observed kinetic heterogeneity in the (M)L214 mutant RCs, although the genuine behavior of these RCs is likely to borrow aspects of both models. Large molecular movements, such as the BA tail conformers observed in the crystal structure of the (M)L214G mutant, may give rise to static heterogeneity among RC populations, as these types of movements would occur much more slowly than the electron transfer events studied here. In addition, the observed lack of continuous electron density at the BA tail in the (M)L214G and (M)L214A structures suggests that more rapid and disordered movements also occur within these proteins, and may influence electron transfer rates on the picosecond and nanosecond timescales. This is because the rapid tail movements may either cause small displacements the H_A macrocycle, or they may favor electron transfer reactions through alternate tunneling pathways which are associated with slower rates of electron transfer. Furthermore, although the (M)214 mutations have little effect on the energetics of the H_A pigment in the ground state, these mutations may give rise to opportunities for cofactor relaxation when entering an anionic state, giving rise to changes in electron transfer rates once $P^+H_A^-$ is attained, as the dynamic model indicates. Whatever the underlying reason accounting for these changes in electron transfer rates, it appears that there is a correlation between the volume of the (M)214 residue, the degree of structural disorder in the B_A tail, and the rate of electron transfer from H_A to Q_A .

4.6 Functional analysis of Zn- and Zn-\beta RCs. I exploited Zn-BChl-containing RCs to evaluate the effects of changing the H_A cofactor coordination state on electron transfer in the RC. This comparison would be impossible in a (Mg²⁺-) BChl-containing RC because the H_A pocket contains BPhe when a fifth ligand is absent (Saer et al. 2013a). Evidently the Zn²⁺ ion is more tightly bound to the bacteriochlorin macrocycle than Mg²⁺, and so Zn-BChl is resistant to whatever mechanism is responsible for the dechelation of BChl to yield BPhe in the H_A pocket (Kobayashi et al. 1998; Saer et al. 2013a; Jaschke and Beatty 2007).

The kinetic consequences of substituting Zn-BChl into the RC in an otherwise wild type background were previously studied, and it was suggested that the Zn-RC functions similarly to the wild type RC (Lin et al. 2009). However, the EADS data (Figure 3.21) obtained as part of this thesis research indicate a slower rate of electron transfer from H_A to Q_A for the Zn-RC. The EADS with a hundreds of picoseconds time constant for the Zn-RC fitted best to ~290 ps, compared to ~210 ps in the wild type RC. The difference between our experimental values and those previously obtained by Lin *et al.* (Lin et al. 2009) likely stems from our use of a broadband detection setup, in contrast to a single wavelength detector. Furthermore, the final charge separation yields of the Zn-RC in our work were also lower, with evidence of some P⁺I_A⁻ recombination in the non-decaying EADS. In this respect, the Zn-RC is more similar to the (M)L214H mutant and Zn- β -RC because the presence of ground state recombination on this time scale indicates that the rate of recombination from P⁺I_A⁻ is increased. The rate of recombination in the Zn-RC was thus calculated based on the

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observed lifetime of the $P^+I_{A^-}$ state and the amount of P^+ remaining at the end of the time window of the experiment according to the formula:

$$\tau_{\rm rec} = \tau_{\rm obs} / ((100 - \phi) / 100)$$
^[2]

where τ_{rec} is the rate of recombination, τ_{obs} is the P⁺I_A⁻ lifetime, and ϕ is the final yield of P⁺ (Kirmaier et al. 1995b). For the Zn-RC, this yielded a value of 1.1 ns, a value closer to that of the (M)L214H mutant and Zn- β -RC (651 ps and 711 ps, respectively) than the 20 ns value obtained for the wild type RC from which Q_A had been removed (Table 3.3).(Parson et al. 1975)

The relatively long-lived hundreds of picoseconds EADS obtained for the (M)L214H mutant from our datasets compared to previously published P⁺I_A⁻ lifetimes suggests a degree of heterogeneity in the P⁺H_A⁻ decay (Kirmaier et al. 1995b; Pawlowicz et al. 2009). When the transient absorption datasets were truncated from 6 ns to a shorter time window (2 ns), the P⁺H_A⁻ decay rates were found to be closer to the previously reported rates. The slower rates observed in the (M)L214H mutant and the Zn- β -RC likely reflect more complex kinetic heterogeneity than can be described by four exponential components, reflecting an average of forward electron transfer as well as recombination to the ground state in RCs in the P⁺I_A⁻ state. A previous analysis of the kinetics of the (M)L214H mutant in which electron transfer to Q_A was blocked demonstrated that the recombination rate from P⁺I⁻ was approximately 1 ns (compared to ~20 ns in the wild type RC (Kirmaier et al. 1995b). Furthermore, the calculated charge recombination rates in the (M)L214H, and Zn- β -RC indicate that charge recombination occurs much faster than in the wild type RC. This difference likely contributes to the relatively shallow fitting minima obtained in the (M)L214H mutant and Zn- β -RC for

this EADS (compare the deviations between the wild type RC/Zn-RC and (M)L214H mutant/Zn- β -RC in the green spectra of Figure 3.22). Because the Zn-RC also has a more rapid rate rate of charge recombination than the wild type RC, I suggest that the presence of the metal center at H_A in the Zn-RC results in an H_A⁻ anion that is closer in energy to P⁺B_A⁻ compared to the wild type RC. This suggestion is consistent with previous mutagenic studies on RCs in which a strong correlation was found between the rate of P⁺H_A⁻ recombination and the similarity of B_A⁻ and H_A⁻ energies (Wang et al. 2012). Clearly, this energetic proximity of H_A⁻ to B_A⁻ is less in the Zn-RC than in the (M)L214H mutant and Zn- β -RC, because the P⁺H_A⁻ recombination rate of the Zn-RC is relatively slow.

In addition to the rate of recombination, it was also possible to extract information on the rates of forward electron transfer from I_A^- to Q_A , by taking into account the lifetime of the hundreds of picoseconds EADS, as well as the final $P^+Q_A^-$ yield of each RC. The rate of electron transfer was calculated according to the equation:

$$\tau_{\rm QT} = \tau_{\rm obs} / (\phi / 100) \tag{3}$$

where τ_{QT} is the rate of electron transfer, τ_{obs} is the P⁺I_A⁻ lifetime, and ϕ is the final yield of P⁺ (Kirmaier et al. 1995b). The values of τ_{QT} are 0.2 ns for the wild type RC, 4 ns ((M)L214H mutant), 0.4 ns (Zn-RC), and 6.4 ns (Zn- β -RC). From these data, it appears that the presence of a fifth coordinate at the H_A metal center is associated with a much reduced rate of forward electron transfer from I_A⁻ to Q_A.

The greater similarity of Zn-RC kinetic behavior to that of the wild type RC, than to the (M)L214H mutant and Zn- β -RC, evidently is because the H_A cofactor is a 4-coordinate Zn-BChl. This indicates that the five-coordinate center present in the latter two RCs is the

key player in the observed low yields of charge separation, and that the presence or absence of a metal in the H_A bacterochlorin is of less consequence. One explanation for the observed results stems from the Q_x transition energies of the H_A cofactors among these RCs. Previous studies on metal-substituted bacteriochlorins in vitro have reported a linear correlation between Qx transition energies, redox potential, and electronegativity (Pauling values) of the bacteriochlorin metal center (Hartwich et al. 1998; Noy et al. 1998). I suggest that the 4coordinate Zn-BChl at H_A functions similarly to a BPhe molecule because the Zn-BChl transition energy, redox potential, and central ion electronegativity are closer to that of BPhe (as indicated by the Q_x aborption peak) than a 5-coordinate BChl or Zn-BChl. In the case of central ion electronegativity, although the Pauling value for Zn^{2+} (~ 1.6) is closer to Mg^{2+} (~ 1.3) than H (~ 2), this value is similar to both and lies between Mg^{2+} and H. Additionally, an increase in the coordination state of the metal center of a bacterochlorin has been associated with a lower electronegativity value for the metal (Hartwich et al. 1998). Thus, a 5coordinate Zn-BChl should be less electronegative than both the 4-coordinate counterpart and BPhe.

In addition to the observed kinetic differences upon entering the $P^+H_A^-$ state, the presence of a metal at H_A imbues different spectroscopic properties on the I_A^- anion. The transient absorption spectra of the $P^+I_A^-$ state for Zn-BChl-containing RCs are more similar to each other (and the (M)L214H mutant) than the wild type RC in the 650 to 730 nm region. As noted in the Results (section 3.9.3), the wild type RC has an absorption maximum at higher energy in this region compared to the other 3 RCs. Thus, the red-shifted peak in these three RCs appears to be correlated with the presence of a metal at H_A , regardless of coordination state or the type of metal present. Interestingly, the RC of the photosynthetic bacterium *Acidiphilium rubrum* contains Zn-BChl in place of BChl, but this organism contains BPhe at H_A. In this organism, Zn-BChl is presumably synthesized by Mg-containing BChl synthesis intermediates (Masuda et al. 1999). After the final step of BChl synthesis, it is thought that the low environmental pH promotes pheophytinization BChl, at which point a Zn²⁺ atom incorporates into the chlorin macrocycle by an unknown mechanism (Masuda et al. 1999). This theoretically provides a pool of both Zn-BChl and BPhe with which the apo-RC can selectively incorporate. This mechanism of Zn-BChl biosynthesis is different from that of the *R. sphaeroides bchD* mutant, because this mutant utilizes Zn-containing synthesis intermediates. Although electron transfer has been studied only to the P⁺H_A⁻ state in *A. rubrum* (Tomi et al. 2007), we speculate that in this RC the rate of electron transfer to Q_A is like that in the wild type RC. Based on our results, it appears that natural selection would favor the presence of BPhe at H_A in evolution because it results in a much slower decay of P⁺I_A⁻ via charge recombination.

4.7 Consequences of the absence of Mg-BChl in RCs. In the ground state absorption spectra of the Zn-RC, as well as in previous low temperature measurements, a single peak was present in the Q_x region near 560 nm, originally attributed to overlapping absorption of 4-coordinate Zn-BChls in the H_{A,B} pockets (Neupane et al. 2012; Lin et al. 2009). However, as reported by Neupane *et al.* (Neupane et al. 2012) and confirmed here, the Zn- β -RC has little or no absorption around 560 nm, whereas it would be thought that only the component due to the H_A Zn-BChl would be shifted as a result of the (M) L214H mutation, as is seen when this mutation is introduced into the wild type RC (Figure 3.18). That is, a ~50% decrease in the ~560 nm peak amplitude was expected, whereas a nearly complete loss of this peak was found. Neupane *et al.* offered three possible explanations for the absence of a

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defined H_B peak in the Q_x region of the Zn- β RC: (i) the Zn-RC and the Zn- β -RC lack an H_B cofactor; (ii) the Zn- β -RC contains two penta-coordinated Zn-BChls at H_{A,B}; (iii) absorption intensity is being redistributed among the bands of the cofactors (a phenomenon known as intensity borrowing .(Orlandi and Siebrand 1973; Neupane et al. 2012)). The Zn-RC and Zn- β -RC crystal structures show that, relative to the wild type RC, the Zn-BChl in the H_B pocket of the Zn-RC is bound at less than full occupancy or is highly disordered, and perhaps the occupancy is even lower or disorder greater in the $Zn-\beta-RC$ (Table 3.3). These conclusions are based on weaker density in omit difference maps of the H_B Zn-BChl, the large B-factors associated with the Zn^{2+} in the H_B pocket, and the low anomalous signal intensities for that Zn^{2+} atom (compare the values for H_B to those of the other cofactors in Table 3.3). Interestingly, the average B-factor of the H_B BPhe from the highest resolution RC structure currently available in the PDB (2J8C) database is greater than all of the other cofactors (Koepke et al. 2007), perhaps indicating weaker binding to this pocket in the wild type RC as well. As mentioned in Section 3.9.2, the B-factor for the protein component surrounding the H_B Zn-BChl is comparable to the average of the structure, consistent with the idea that the RC is a relatively rigid protein, and does not rearrange its folding due to changes in cofactor composition or even when a cavity is created by the lack of a cofactor, as was observed in pigment exclusion experiments on the H_B BPhe (Jones 2008; Watson et al. 2005a). The Q_x larger spectral bandwidth of the H_B cofactor compared to H_A also supports a greater H_B site heterogeneity (Figure 3.18). Additional evidence for relatively weak binding of BPhe in the wild type RC H_B pocket comes from experiments involving substitution of BPhe with plant pheophytin (as well as a variety of other pigments). These experiments demonstrated that substitution is preferential in the H_B over the H_A site, consistent with a weaker binding of

BPhe in the H_B pocket (Meyer and Scheer 1995; Franken et al. 1997). In vitro, the binding pockets for H_{A,B} are selective for pheophytins, as incubation of RCs in a molar excess of metal-containing bacteriochlorins (the standard procedure for pigment exchange) resulted in substitution of pigments at the $B_{A,B}$ binding pockets instead of $H_{A,B}$ (Struck and Scheer 1990; Scheer and Hartwich 1995; Jones 2008). The greater hydrophobicity of BPhe over BChl and Zn-BChl, as indicated by the longer retention time for BPhe in reversed-phase HPLC using a C18 column (Jaschke and Beatty 2007), may be part of the reason why binding of metalcontaining bacteriochlorins to the $H_{A,B}$ pockets is weak, although it is not clear why the H_B Zn-BChl may be lost more readily or less efficiently inserted into the RC than the HA Zn-BChl. Alternatively, it is possible that the Zn-RCs contain partially occupied H_B sites due to a lack of a sufficient quantity of available Zn-BChls in the cell. One important consequence of the *bchD* mutation yielding Zn-BChl-producing strains of *R. sphaeroides* is the severely impaired ability to synthesize the pigment (Jaschke and Beatty 2007; Jaschke et al. 2011). Therefore the inability of the organism to grow phototrophically is because of a paucity in the cellular content of light-harvesting and RC complexes (Jaschke and Beatty 2007).

Unlike the H_B cofactors of the Zn-RC and Zn- β -RC, the H_A cofactors appear less disordered, suggesting there is no issue with the occupancy of the pocket. From a structural perspective, the H_A cofactors appear to assume a similar configuration in the two Zn-BChl-containing RCs (Fig. 3.19), with a possible macrocycle displacement of 0.6 Å between the two RCs. One cannot reach a firm conclusion on this geometric displacement between the cofactors because of the poor resolution of the data obtained for these two RCs; however the small degree of displacement is consistent with the interpretation that the differences in the

functional characteristics of the two Zn-BChl-containing RCs stem predominantly from changes in H_A cofactor coordination state.

4.8 Biological implications of unnatural cofactor incorporation. Our time-resolved data demonstrate that in all 4 of these RCs $P^+I_A^-$ is achieved rapidly (within a few ps), and that differences between RCs become apparent after this state is attained. The decay kinetics of the $P^+H_A^-$ transient at 675 nm indicate that this state is relatively long-lived in the Zn- β -RC, as well as in the (M)L214H mutant, in agreement with previous results (Kirmaier et al. 1991; Kirmaier et al. 1995a, b). Furthermore, the global analysis indicates a similar degree of special pair charge recombination associated with this long-lived $P^+H_A^-$ state in the Zn- β -RC, much like in the (M)L214H mutant. The charge separation yields are consistent with this interpretation (*i.e.*, the (M)L214H mutant and the Zn- β -RC yields were 14% and 10% P⁺Q_A⁻, respectively, whereas the wild type RC and Zn-RC yields were 100% and 74% $P^+Q_A^-$, respectively). These results indicate that RCs have a degree of functional tolerance for different cofactors, and that the protein component is a key instrument in the design of an efficient charge separation pathway. The Zn-RC studied here is a case in point, as it contains a cofactor arrangement supportive of a faster rate of ground state recombination from the $P^+I_A^-$ state, but nevertheless is capable of attaining a relatively high charge separation efficiency. This likely is because the protein scaffold of the Zn-RC has remained unchanged from the wild type RC counterpart, granting this mutant RC the same favorable cofactor geometries and tunneling pathways available in the wild type RC. From an evolutionary perspective, this could be advantageous to photosynthetic organisms during periods of environmental change, such as a change in the wavelengths of light available, for it would allow for the continued utilization of existing protein machinery when the production of a

new type of (bacterio)chlorin would provide a selective advantage. In such a case, the new pigment could be incorporated into a protein "template" with suitable geometries, which would grant the organism some degree of charge separation capability, enabling photosynthetic growth. Mutations in protein side chains may later provide further energetic optimization, resulting in a high-efficiency RC that assembles with a new pigment. For this to happen, however, the photosynthetic organism must be able to survive for some time with an inefficient RC to allow beneficial mutations to accumulate. The results from Section 3.4 show that LH2-lacking *R. sphaeroides* strains containing RCs with small-nonpolar side chains at H_A retain their ability to grow at low photon fluxes, despite the relatively low efficiencies of these RCs (compared to the wild type RC), indicating that purple photoheterotrophs are indeed quite capable of surviving with lower efficiency RCs.

4.9 Why do natural type II RCs contain a BPhe in the H_A **or analogous pocket, regardless of the presence of Mg**²⁺ **versus Zn**²⁺ **in their (B)Chls?** The type II RC found in many anoxygenic phototrophs, as well as all species of cyanobacteria and photosynthetic eukaryotes, contains a (B)Phe molecule as an intermediate electron acceptor followed by a quinone molecule (Blankenship 2010). The use of this pigment is rather interesting, because type I RCs such as photosystem I of oxygenic phototrophs and the RCs of green sulfur bacteria and heliobacteria lack such a pigment, yet function with high quantum efficiencies (Chauvet et al. 2013). Why, then, has evolution chosen pheophytins as intermediate electron acceptors in type II RCs when other RCs that don't require pheophytin exist?

Regardless of the subsequent uses of captured light energy, charge separation in the RC relies on the separation of an electron-hole pair. This result is ultimately accomplished

using a cofactor cascade capable of reducing some entity (quinone or ferredoxin) which can physically remove the electron from the RC to prevent recombination. For this to happen, the rate of recombination between the diffusible electron acceptor and primary donor must be slow enough to allow for the electron acceptor to dissociate from the protein; and so the relatively slow rate of recombination is obtained by a sufficient number of electron transfer intermediates to create a large distance between the terminal electron acceptor and the primary donor. Because the back reaction does not involve the electron transfer intermediates (it can occur only between the oxidized donor and an anionic radical), creating a large distance between the donor and acceptor significantly decreases the rate of the back reaction (Moser et al. 1992). I suggest that exclusive use of (B)Chl as electron transfer intermediates in type II RCs would not generate a sufficient energy barrier between the intermediates for electron transfer to proceed efficiently (essentially unidirectionally) in the forward direction. Nature has solved this problem by using a quinone (Q_A) as an electron transfer intermediate in type II RCs. The use of a quinone, however, results in a driving force for forward electron transfer that would be too large if (B)Chl were used as the electron donor. Instead of having to synthesize a novel compound with the appropriate energetic properties, Mg²⁺ is removed from some of the (B)Chl, generating (B)Phe that can be reduced by a (B)Chl as well as efficiently transfer an electron to a quinone.

The energetic landscape of the RC's photoactive pigments only partly answers the question about the use of (B)Phe in type II RCs, however. Studies in which hydrogen bonds are introduced into or removed from the BChls of the RC from *R. sphaeroides* have shown that the electron transfer properties of the pigment are highly tunable by its protein environment (Mattioli et al. 1995; Mattioli et al. 1994). Thus it should be feasible to simply

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tune a (B)Chl with the surrounding protein component for the (B)Chl to act similarly to (B)Phe in the context of electron transfer (although this has yet to have been performed). Furthermore, the use of different quinones in the RC can achieve a similar effect (Gunner and Dutton 1989), although the use of multiple quinones in an electron transfer cascade has not been observed in RCs in nature. The fact that an H_A BChl in the (M)L214H mutant RC has similar energetic properties to the B_A BChl suggests that multiple mutations must be made over evolutionary timescales to properly tune the H_A BChl for efficient electron transfer (Kirmaier et al. 1995b). If a different quinone were to be used, then an ensemble of new biosynthetic genes would be needed for the organism to make the novel molecule, as well as modification of all the quinone-utilizing proteins for the cell to function properly with the different quinone molecule.

5. SUMMARY AND FUTURE DIRECTIONS

The work presented here was begun with the intention of advancing the understanding of the function of the RC of *Rhodobacter sphaeroides*, a model photosynthetic organism. A mutagenic approach was employed to change the environment of the photoactive BPhe molecule located in the H_A binding pocket, to investigate questions relating to how a BPhe comes to be present in this pocket, instead of the BChl present in other RC bacteriochlorin-containing pockets. The results show that: (i) BChl incorporation into H_A requires the presence of fifth coordinate that may be provided by a histidine, asparagine or glutamine side chain ligand; (ii) the large side chain of a leucine residue at (M)214 is important in maintaining the positioning of the B_A phytyl tail, as well as (iii) maintaining a fast electron transfer rate from H_A to Q_A and (iv) a slow rate of recombination from $P^+H_A^-$ to the ground state. The interplay between (M)214 and electron transfer is likely to be the result of structural perturbations, giving rise to a variety of static populations of RCs that result in decreased electron transfer rates, as well as increasing opportunities for relaxation of the H_A^- state, thereby increasing the rate of recombination from $P^+ H_A^-$ to the ground state.

One key unknown that remains in spite of the results of this work is the remarkable ability of the (M)L214G mutant strain to grow photoheterotrophically despite its RC having a lower yield of charge separation than the (M)L214H mutant, which suffers a detriment to photoheterotrophic growth. Although the (M)L214G mutant strain does not suffer a penalty in photoheterotrophic growth, no natural (M)L214G mutants were found in the 100-organism sequence alignment. It would be interesting to study whether the (M)L214G RC incurs the same structural perturbations in the native membrane that were observed *in vitro*. Because this mutant RC is associated with changes in the absorption spectrum, spectroscopic studies on the chromatophores of the (M)L214G mutant lacking LH1 could provide insight into the behavior of this RC *in vivo*.

Throughout this work, attempts were made to elucidate the origin of the molecule BPhe. However the precise locus or mechanism of the origin of BPhe remains a mystery. Based on my work, it appears that the RC does not act as a dedicated de-chelator of BChl, and that axial ligands to BChl provide the selectivity that distinguishes a BChl-binding pocket from a BPhe-binding pocket. The isolation of a mutant that is unable to produce BPhe would provide valuable information on this molecule's origin. Alternatively, it is possible that BChl self-pheophytinizes during RC assembly due to the lack of a fifth coordinate. The *de novo* assembly of a functional RC using a cell-free system could resolve this question, as one could theoretically control the pigment input into the reaction mixture (*i.e.*, add only BChl to a reaction mixture and see if BPhe is incorporated into the RC). Although bacterial light-harvesting complexes have been assembled from purified proteins and BChl (Recchia et al. 1998; Schwarz et al. 2008), membrane protein complex assembly *in vitro* is largely an emergent technology, and so attempts to employ these methods on the RC would be challenging.

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Mutant	Average slope of growth curve*	Standard deviation
Wild type	0.54	0.04
∆puhA	No growth	No growth
(M)L214G	0.47	0.16
(M)L214A	0.51	0.01
(M)L214C	0.49	0.22
(M)L214V	0.44	0.10
(M)L214I	0.68	0.18
(M)L214M	0.56	0.08
(M)L214N	0.13	0.03
(M)L214Q	0.11	0.06
(M)L214H	0.07	0.01

APPENDIX 1. Tabulated photoheterotrophic growth rates of wild type and (M)214 mutants at 5 μ E·m⁻²·s⁻¹ light intensity.

*In Klett units per hour. One Klett unit is approximately equal to 3×10^6 cells per mL.

APPENDIX 2. X-ray data collection and refinement statistics for (M)L214G, (M)L214A, and (M)L214N RCs.

Structure		(M)L214G	(M)L214A	(M)L214N	
Accession Code		4IN5 4IN6		4IN7	
Unit Call Parameters	a = b	139.11 Å	139.03 Å	139.14 Å	
Unit Cen l'arameters	С	184.69 Å	184.34 Å	185.14 Å	
Resolution (Å)*		60.24 - 2.20 (2.32 - 2.20)	60.00 - 2.70 (2.75 - 2.70)	38.54 – 2.85 (3.00 – 2.85)	
No. of reflections**		579188 (105008)	330265 (56265)	353391 (48933)	
R _{merge} *		0.085 (0.590)	0.154 (0.545)	0.098 (0.498)	
Ι/σΙ*		11.3 (2.8)	25.7 (3.3)	17.2 (4.3)	
Multiplicity*		5.5 (5.4)	5.9 (3.9)	7.2 (7.3)	
Completeness* (%)		99.9 (100.0)	98.5 (97.8)	99.9 (99.9)	
R _{work}		0.191	0.189	0.179	
R _{free}		0.212	0.225	0.207	
Wilson B-factor (Å ²)		38.8	53.2	60.9	
Overall B-factor (Å ²)		40.8	41.2	42.5	
Bond length rmsd (Å)		0.018	0.019	0.020	
Bond angle rmsd (°)		1.91	2.18	2.27	

* Numbers in parentheses reflect statistics for the highest resolution shell.

** Number in parentheses corresponds to number of unique reflections

Structure		Zn-RC	Zn-β-RC				
Accession Code							
Unit Call Daramatara	a = b	139.45 Å	139.59 Å				
Unit Cen Farameters	С	184.18 Å	184.05 Å				
Resolution (Å)*		73.23 - 2.85	69.79 - 2.85				
		(2.90 – 2.85)	(2.90 – 2.85)				
R _{merge} *		0.105 (0.972)	0.098 (0.705)				
I/σI*		10.5 (1.5)	12.6 (2.4)				
Multiplicity*		6.2 (4.7)	7.0 (6.2)				
Completeness* (%)		96 (95)	87.4 (85.6)				
Rwork		0.182	0.167				
R _{free}		0.242	0.216				
Wilson B-factor (Å ²)		53.1	47.0				
Overall B-factor (Å ²)		58.2	54.8				
Coordinate error** (Å	()	0.21	0.17				
Bond length rmsd (Å))	0.011	0.013				
Bond angle rmsd (°)		2.6	2.7				

APPENDIX 3. X-ray data collection and refinement statistics for the Zn- and Zn- β -RCs.

* Numbers in parentheses reflect statistics for the highest resolution shell.

**Coordinate error is the estimated standard uncertainty from maximum likelihood refinement.

APPENDIX 4: Multiple sequence alignment of the PufM protein sequence

Accession No.*	Position	<u>P</u>
WP_002720420_1	181	WT <mark>NN</mark> FSLVH <mark>GNLFYNPFH</mark>
BAC54028 1	181	WTNNFSLVHGNLFYNPFH
CAR63836 1	181	WTNNFSLVHGNLFYNPFH
WP 008028573 1	181	WTNMFSLTYGNLFYNPFH
YP 003576866 1	179	WTNQ <mark>FSL</mark> DH <mark>GNLFYNPFH</mark>
AEW50177 1	179	WTNQ <mark>FSLDH</mark> GNLFYNPFH
AAM48658 1	180	WTNLFSLTYGNLFYNPFH
WP 008231901 1	181	WTNLFSITYGNLFYNPFH
YP_006965282_1	181	WT <mark>NL</mark> FSLTYGNLFYNPFH
AAX48162_1	181	WT <mark>NL</mark> FSLTYGNLFYNPFH
WP 009159933 1	181	WT <mark>NL</mark> FSLTYGNLFYNPFH
WP 009831029 1	181	WT <mark>NL</mark> FSLTYGNLFYNPFH
WP_007206416_1	181	WT <mark>NL</mark> FSITH <mark>GNLFYNPFH</mark>
WP_019954423_1	181	WT <mark>NL</mark> FSITH <mark>GNLFYNPFH</mark>
WP_007164992_1	181	WT <mark>NN</mark> FSLIH <mark>GNLFYNPFH</mark>
BAA76948_1	180	WT <mark>T</mark> AFSIRYGNLYYNPFH
BAC54030_1	181	WT <mark>NN</mark> FSLTH <mark>GNLFYNPFH</mark>
WP_008280383_1	181	WT <mark>T</mark> AFSIRYGNLYYNPFH
WP_009819265_1	181	WT <mark>T</mark> AFSIRYGNLYYNPFH
WP_022573914_1	181	WT <mark>T</mark> AFSIRYGNLYYNPFH
CAR63830_1	156	WT <mark>NN</mark> FSLVH <mark>GNLFYNPFH</mark>
AAY85889_1	181	WT <mark>T</mark> AYSIRYGNLYYNPFH
WP_007814239_1	181	WT <mark>T</mark> AFSIRYGNLYYNPFH
CAR63832_1	181	WT <mark>NM</mark> FSLTYGNLFYNPFH
WP_008962605_1	181	WT <mark>NN</mark> FSITH <mark>GNLFYNPFH</mark>
WP_006615516_1	181	WT <mark>NN</mark> FSITH <mark>GNLFYNPFH</mark>
YP_001203741_1	181	WT <mark>NN</mark> FSITH <mark>GNLFYNPFH</mark>
WP_020044323_1	181	WT <mark>TAFSIRYGNL</mark> YNPFH
YP_001534858_1	181	WTTAFSIRYGNLYYNPFH
YP 007515475 1	181	WT <mark>NN</mark> FSI <mark>TH</mark> GNLFYNPFH

Protein Sequence**

WΤ	'NNFS	LVH	GNLE	FYNPI	ΞĦG	LSIA	FLYG	GSAI	LFAM	IHGAT	ILAV	'SRF	GGEF	REL	EQI	ADRO	GT2
WΤ	NNFS	SLVH	GNLE	FYNPI	ΉG	LSIA	FLYG	SSAI	LFAM	IHGAT	ILAV	'SRF	GGVF	REL	EQI	ADRO	GT <i>I</i>
WΤ	NNFS	SLVH	GNLE	FYNPI	ΉG	LSIA	FLYG	SSAI	LFAM	IHGAT	ILAV	'SRF	GGEF	REL	EQI	ADR	SRĢ
WΤ	NMFS	SLTY	GNLE	FYNPI	ΉG	LCIG	FLYG	SSAI	LFAM	IHGAT	IIAV	'SRF	GGEF	REL	EQM	VDRO	GTA
WΤ	NQFS	LDH	GNLE	FYNPI	ΞĦG	LSIA	ALYO	SSAI	LFAM	IHGAT	ILAV	TRF	GGEF	REL	EQIV	VDRO	GTA
WΤ	NQFS	LDH	GNLE	FYNPI	ΞĦG	LSIA	ALYO	SSAI	LFAM	IHGAT	ILAV	TRF	GGEF	REL	EQIV	VDRO	GTA
WΤ	NLFS	SLTY	GNLE	FYNPI	ΗA	LSIA	FLYO	SSAI	LFAM	IHGAT	ILAV	SRY	GGEF	REI	EQI	VDRO	GT <i>I</i>
WΤ	NLFS	SITY	GNLE	FYNPI	ΗA	LSIV	FLYG	SSAI	LFAM	IHGAT	ILAV	'SRF	GGEF	REI	EQI	VDRO	GTZ
WΤ	NLFS	SLTY	GNLE	FYNPI	ΉA	LSIV	FLYO	SSAI	LFAM	IHGAT	ILAV	SRY	GGEF	REI	EQIV	VDRO	GTA
WΤ	NLFS	SLTY	GNLE	FYNPI	ΉA	LSIA	FLYG	SSAI	LFAM	IHGAT	ILSV	TRL	GGDF	REL	EQIV	VDRO	GT <i>I</i>
WΤ	NLFS	SLTY	GNLE	FYNPI	ΉA	LSIA	FLYG	SSAI	LFAM	IHGAT	ILAV	SRY	GGEF	REI	EQIV	VDRO	GT <i>I</i>
WΤ	NLFS	SLTY	GNLE	FYNPI	ΉA	LSIA	FLYG	SSAI	LFAM	IHGAT	ILAV	SRY	GGEF	REI	EQIV	VDRO	GT <i>I</i>
WΤ	NLFS	SITH	GNLE	FYNPI	ΉA	LSIA	FLYG	SSVI	LFAM	IHGAT	ILAV	'SRF	GGEF	REI	EQIV	VDRO	GΤΖ
WΤ	NLFS	SITH	GNLE	FYNPI	ΗA	LSIV	FLYG	SSVI	LFAM	IHGAT	ILAV	'SRF	GGEF	REI	EQIV	VDRO	GΤΖ
WΤ	'NNFS	SLIH	GNLE	FYNPI	ΗA	LSIV	FLYG	GSA <mark>v</mark>	LFAM	IHGAT	ILAV	GRΥ	GGEF	REI	EQI	r DR(GΤΖ
WΤ	TAFS	SIRY	GNL	YNPI	ΉA	LSIA	FLYG	SSAI	LFAM	IHGGT	ILAV	TRF	GGDF	REL	EQI	Y DR(GT <i>I</i>
WΤ	'NNFS	LTH	GNLE	FYNPI	ΗG	LSIA	FLYG	SST <mark>№</mark>	(LFAM	IHGAT	ILAV	SRL	GGEF	REL	EQIV	VDRO	GΤΖ
WΤ	TAFS	SIRY	GNL	YNPI	HC	LSIV	FLYG	SSVI	LFAM	IHGAT	ILAV	TRF	GGDF	REL	EQIV	VDRO	GΤΖ
WΤ	TAFS	SIRY	GNL	YNPI	HC	LSIV	FLYG	SSVI	LFAM	IHGAT	ILAV	SRF	GGDF	REL	EQI	VDRO	GTZ
WΤ	TAFS	SIRY	GNL	YNPI	ΉA	LSIV	FLYO	SSVI	LFAM	IHGAT	ILAV	TRF	GGDF	REL	EQI	YDRO	GΤZ
WΤ	NNFS	LVH	GNLE	FYNPI	ΉG	LSIA	FLYO	SSAI	LFAM	IHGAT	ILAV	'SRF	GGEF	REL	EQI	ADRO	GΤZ
WΤ	TAYS	SIRY	GNL	YNPI	ΉA	LSIV	FLYO	SSVI	LFAM	IHGGT	ILAT	TRF	GGDF	REL	EQI	YDRO	GΤZ
WΤ	TAFS	SIRY	GNL	YNPE	HC	LSIV	FLYO	SSVI	LFAM	IHGAT	ILAV	TRF	GGDF	REL	EQI	Y DRO	GTZ
WΤ	NMFS	SLTY	GNLE	FYNPI	ΗA	LSIA	FLYG	5SA <mark>I</mark>	LFAM	IHGAT	ILAV	SPL	RRRA	4			
WΤ	'NNFS	SITH	GNLE	TYNPI	ΗM	LSIA	FLYG	SSAI	LFAM	IHGAT	VLAI	SRY	GGEF	REL	EQIV	VDRO	GT <i>I</i>
WΤ	'NNFS	SITH	GNLE	TYNPI	ΗM	LSIA	FLYG	SSAI	LFAM	IHGAT	VLAI	SRY	GGEF	REL	EQIV	VDRO	GT <i>I</i>
WΤ	NNFS	SITH	GNLE	TYNPI	ΗM	LSIA	FLYG	SSAI	LFAM	IHGAT	VLAI	SRY	GGEF	REL	EQI	VDRO	GT2
WΤ	TAFS	SIRY	GNLY	YNPI	THC	LSIV	FLYC	GSVI	LFAM	IHGAT	ILAT	TRF	GGDF	REL	EQI	YDRO	GT2
WΤ	TAFS	SIRY	GNLY	YNPI	ΉA	LSIV	FLYC	GSVI	LFAM	IHGAT	ILAV	TRF	GGDF	REL	EQI	YDR(GT <i>I</i>
WΤ	NNFS	SITH	GNLF	TYNPI	ЪĤМ	LSIA	FLYG	SSAI	LFAM	IHGAT	VLAI	SRY	GGEF	REL	EQI	VDRO	GT <i>I</i>

WP_017669607_1	181	WT <mark>AAFSIRYGNLFYNPFHA</mark> LSIAFLYGS <mark>TL</mark> LFAMHGATILAV <mark>GRYGGEREIEQI</mark> YDRGTA
BAA22642_1	181	WT <mark>NN</mark> FSLTYGNLFYNPFH <mark>G</mark> LSIAFLYGS <mark>AL</mark> LFAMHGATILAVSRFGGDRELEQIVDRGTA
WP_009758337_1	181	WT <mark>NN</mark> FSLTYGNLFYNPFH <mark>A</mark> LSIVFLYGS <mark>AL</mark> LFAMHGATILAVSRYGGEREIEQI <mark>T</mark> DRGTA
WP_009031272_1	181	WT <mark>NN</mark> FSITHGNLFYNPFH <mark>M</mark> LSIAFLYGS <mark>AL</mark> LFAMHGATVLAISRYGGERELEQIVDRGTA
YP_005438200_1	181	WT <mark>A</mark> AFSLRYGNLFYNPFH <mark>A</mark> LSIAFLYG <mark>AT<mark>L</mark>LFAMHGATILAVSRFGGERELEQI<mark>A</mark>DRGTA</mark>
AA093122_1	181	WT <mark>A</mark> AFSLRYGNLFYNPFH <mark>A</mark> LSIAFLYG <mark>AT<mark>L</mark>LFAMHGATILAVSRFGGERELEQI<mark>A</mark>DRGTA</mark>
WP_020042497_1	181	WT <mark>TAFSIRYGNLYYNPFH</mark> CLSIVFLYGSC <mark>L</mark> LFAMHGATILAVSRFGGDRELEQIVDRGTA
WP_010236075_1	181	WT <mark>NN</mark> FSLIYGNLFYNPFH <mark>A</mark> LSIAFLYGS <mark>AL</mark> LFAMHGCTILAVSRYGGEREIEQI <mark>T</mark> DRGTA
WP_019903654_1	181	WT <mark>AAFSLRYGNLFYNPFH</mark> MLSIVFLYGS <mark>TL</mark> LFAMHGATILAVSRFGGEREIDQIVDRGTA
YP_001242246_1	181	WT <mark>NNFSI</mark> THGNLFYNPFH <mark>M</mark> LSIAFLYGS <mark>AL</mark> LFAMHGATVLAISRYGGERELEQIVDRGTA
WP_008296327_1	181	WT <mark>AAFSLRYGNLFYNPFH</mark> MLSIAFLYGS <mark>TL</mark> LFAMHGATILAVTRYGGDREIDQITDRGTA
WP_018406831_1	180	WT <mark>AAFSIRYGNLFYNPFH</mark> MLSIAFLYGS <mark>AL</mark> LFAMHGATILAV <mark>GRFGGEREIEQI</mark> IDRGTA
YP_508114_1	180	WT <mark>TAFSIRYGNLYYNPFH</mark> CLSI ^V FLYGS <mark>VL</mark> LFAMHGATILAVSRYGGDRELEQIVDRGTA
WP_018043295_1	181	WT <mark>AAFSLRYGNLFYNPFHM</mark> LSI <mark>VFLYGS</mark> T <mark>L</mark> LFAMHGATILAVSRFGGEREIDQIVDRGTA
WP_019916989_1	181	WT <mark>A</mark> AFSIRYGNLFYNPFH <mark>M</mark> LSIAFLYGS <mark>TL</mark> LFAMHGATILAVSRFGGEREIEQIVDRGTA
WP_020165686_1	181	WT <mark>A</mark> AFSIRYGNLFYNPFH <mark>M</mark> LSIAFLYGS <mark>TL</mark> LFAMHGATILAVSRFGGEREIEQIVDRGTA
WP_008064766_1	181	WT <mark>A</mark> AFSIRYGNLFYNPFH <mark>M</mark> LSIAFLYGS <mark>TL</mark> LFAMHGATILAVSRFGGEREIEQIVDRGTA
YP_001770519_1	181	WT <mark>AAFSLRYGNLFYNPFH</mark> MLSIVFLYGS <mark>TL</mark> LFAMHGATILA <mark>T</mark> SRYG <mark>SEREIEQIVDRGTA</mark>
YP_005449250_1	181	WT <mark>NN</mark> FSI <mark>TH</mark> GNLFYNPFH <mark>M</mark> LSIAFLYGS <mark>AL</mark> LFAMHGATILAVSRYGGEREIEQIVDRGTA
WP_022677059_1	181	WT <mark>A</mark> AFSIRYGNLFYNPFH <mark>CF</mark> SIAFLYGS <mark>TL</mark> LFAMHGATILAV <mark>G</mark> RYGGEREIEQI <mark>T</mark> DRGTA
WP_009019700_1	181	WT <mark>AAFSLRYGNLFYNPFH</mark> MLSIAFLYGS <mark>TL</mark> LFAMHAGTILA <mark>T</mark> SRFGGDREIDQITDRGTA
WP_006787498_1	181	WT <mark>TAFSLRYGNLFYNPFH</mark> MLSIAFLFGS <mark>AL</mark> LFAMHGATILAVTRFGGDRELEQITDRGTA
WP_009021756_1	181	WT <mark>AAFSLRYGNLFYNPFH</mark> MLSIVFLYGS <mark>TL</mark> LFAMHGATILAVTRYGGDREIDQITDRGTA
WP_010405242_1	181	WT <mark>AAFSIRYGNLFYNPFH</mark> CLSIVFLYGS <mark>TL</mark> LFAMHGATILAV <mark>GRYGGERELE</mark> HITDRGTA
WP_018411947_1	181	WT <mark>AAFSIRYGNLFYNPFHM</mark> LSIAFLYGS <mark>TL</mark> LFAMHGATILAVSRFGGEREIEQIVDRGTA
WP_021076706_1	181	WT <mark>NN</mark> FSI <mark>TH</mark> GNLFYNPFH <mark>M</mark> LSIAFLYGS <mark>AL</mark> LFAMHGATILAVSRYGGEREIEQIVDRGTA
WP_008944427_1	181	WT <mark>AAFSIRYGNLYYNPFH</mark> CLSIVFLYGS <mark>VL</mark> LFAMHGATILAV <mark>GKYGGERELEQI</mark> TDRGTA
WP_017664717_1	181	WT <mark>NNFSLTYGNLFYNPFH</mark> ALSIVFLYGS <mark>AV</mark> LFAMHGATILALG <mark>RYGGEREIEQI</mark> TDRGTA
WP_010184133_1	181	WT <mark>AAFSIRYGNLFYNPFH</mark> CLSIVFLYGS <mark>TL</mark> LFAMHGATILAV <mark>GRYGGERELE</mark> HITDRGTA
WP_019012961_1	181	WT <mark>AAFSIRYGNLFYNPFH</mark> ALSIVFLYGS <mark>TL</mark> LFAMHGATILAVSRYGGEREIEQTLDRGTA
WP_020086078_1	181	WT <mark>NQL<mark>SLDYGNLFYNPFH</mark>MLSIAFLYGS<mark>AL</mark>LFAMHGATVLAISRYGGDRELEQITDRGTA</mark>
YP_004110280_1	181	WT <mark>NNFSIKYGNLYYNPFH</mark> CLSIAFLYGS <mark>AL</mark> LFAMHGATILAVSRYGGEREIEQMLDRGTA
YP_570871_1	181	WT <mark>NN</mark> FSIKYGNLYYNPFH <mark>C</mark> LSIAFLYGS <mark>AL</mark> LFAMHGATILAVSRYGGEREIEQMLDRGTA
YP_002362361_1	181	WT <mark>AAFSIRYGNLFYNPFH</mark> MLSI <mark>VFLYGS</mark> T <mark>L</mark> LFAMHAATILAVSRFGGEREIEQ <mark>A</mark> VDRGTA
WP_020503997_1	181	WT <mark>AAFSIAYGNLYYNPFH</mark> MLSIAFLYGS <mark>AV</mark> LFAMHGGTILAVSRYGGDREIDQI <mark>T</mark> DRGTA
BAA25552_1	181	WT <mark>VSFSLRYGNLYY</mark> DPFH <mark>MLSI</mark> VFLYGS <mark>VL</mark> LFAMHGATILAVSRFGGEREVEQTVDRGTA

YP_487598_1	181	WT <mark>NNFSIKYGNLYYNPFH</mark> CLSIAFLYGS <mark>A</mark>	LFAMHGATILAVSRYGGEREIEQMLDRGTA
YP_001925549_1	181	WT <mark>AAFSL</mark> RYGNLFYNPFH <mark>M</mark> LSI <mark>V</mark> FLYGST	LFAMHGCTILA <mark>T</mark> SRYGAEREIDQIVDRGTA
WP_010162300_1	181	WT <mark>AAFSIRYGNLFYNPFH</mark> C <mark>LSI</mark> VFLYGST	LFAMHGATILAV <mark>G</mark> RYGGEREIE <mark>H</mark> ITDRGTA
WP_006724825_1	181	WT <mark>AAFSIRYGNL</mark> YYNPFH <mark>CLSI</mark> VFLYGSV	<mark>L</mark> LFAMHGATILAV <mark>G</mark> KYGGERELEQI <mark>T</mark> DRGTA
NP_946876_1	181	WTNN <mark>FSIKYGNLYYNPFH</mark> CLSIAFLYGS <mark>A</mark>	LFAMHGATILAVSRYGGEREIEQ ^{ML} DRGTA
WP_007196596_1	179	WT <mark>AAFSIRYGNLYYNPFH</mark> C <mark>LSI</mark> VFLYGSV	LFAMHGATILAVTRYGGERELEQITDRGTA
YP_002421731_1	181	WT <mark>AAFSL</mark> RYGNLFYNPFH <mark>M</mark> LSI <mark>VFLYGS</mark> T	LFAMHGCTILA <mark>T</mark> SRYGAEREIDQIVDRGTA
YP_002298302_1	181	WT <mark>A</mark> AFSIRYGNLFYNPFH <mark>A</mark> LSIAFLYGST	LFAMHGATVLATSRFGAEREVEQIVDRGTA
YP_002963963_1	181	WT <mark>AAFSL</mark> RYGNLFYNPFH <mark>MLSI</mark> VFLYGST	LFAMHGGTILA <mark>T</mark> SRYGAEREIDQIVDRGTA
YP_003068997_1	181	WT <mark>AAFSL</mark> RYGNLFYNPFH <mark>MLSI</mark> VFLYGST	LFAMHGGTILA <mark>T</mark> SRYGAEREIDQIVDRGTA
YP_001755478_1	181	WT <mark>AAFSL</mark> RYGNLFYNPFH <mark>MLSI</mark> TFLYGST	LFAMHGGTILACSRYGAEREIDQIVDRGTA
YP_001640199_1	181	WT <mark>AAFSL</mark> RYGNLFYNPFH <mark>MLSI</mark> VFLYGST	LFAMHGGTILA <mark>T</mark> SRYGAEREIDQIVDRGTA
WP_008189646_1	180	WT <mark>AAFSIRYGNLYYNPFH</mark> CLSIVFLYGSV	<mark>L</mark> LFAMHGATILAV <mark>G</mark> KYGGERELEQI <mark>T</mark> DRGTA
WP_017486011_1	181	WT <mark>AAFSLRYGNLFYNPFH</mark> MLSIVFLYGST	LFAMHGGTILA <mark>T</mark> SRYG <mark>SEREID</mark> QIVDRGTA
WP_022723276_1	181	WTNNFSIKYGNLYYNPFHCLSIAFLYGS <mark>A</mark>	<mark>L</mark> LFAMHGATILAVSRYGGEREIEQ ^{ML} DRGTA
WP_022680670_1	180	WT <mark>A</mark> AFSIRYGNLFYNPFH <mark>A</mark> LSIAFLYGST	LFAMHGATILAV <mark>G</mark> RYGGEREIEQI <mark>T</mark> DRGTA
YP_680524_1	181	WTTAFSIRYGNLYYNPFHCLSIVFLYGSV	LFCMHGGTILAV TRYGGDRELEQIYDRGTA
WP_020091995_1	181	WT <mark>AAFSL</mark> RYGNLFYNPFH <mark>MLSI</mark> TFLYGST	LFAMHGCTILACSRYGAEREIDQIVDRGTA
P26279_1	181	WTTAFSIRYGNLYYNPFHCLSIVFLYGSV	LFCMHGGTILAVTRYGGDRELEQIYDRGTA
WP_007563666_1	181	WT <mark>AAFSL</mark> RYGNLFYNPFH <mark>MLSI</mark> TFLYGST	LFAMHGGTILACSRYGADREIDQIVDRGTA
YP_531181_1	180	WTNNFSIKYGNLYYNPFHCLSIAFLYGSA	LFAMHGATILAVSRYGGEREIEQVVDRGTA
WP_007421829_1	181	WTVAFSLRYGNLYYDPFHMLSIVFLYGSV	LFAMHGATILAVSRFGGEREVEQTVDRGTA
YP_003819968_1	181	WTGAFSITYGNLFYNPFHCLSIVFLYGST	LFAMHGATILATSRYGAEREVEQITDRGTG
WP_007039476_1	181	WT <mark>AAFSIRYGNLYYNPFH</mark> MLSIAFLYGSA	LFAMHGATIL <mark>SVSRFGGDREID</mark> QIVDRGTA
CBM41421_1	181	WTNLFSLTYGNLFYNPFHALSIVFLYGSA	LFAMHGATILAVSRLGGDRELEQIVDRGTA
YP_004284102_1	181	WTVAFSLRYGNLYYDPFHMLSIVFLYGSV	LLFAMHGATILAVSRFGGEREVEQTVDRGTA
WP_010682616_1	181	WTAAFSLRYGNLFYNPFHMLSITFLYGST	LFAMHGGTILACSRYGAEREIDQIVDRGTA
WP_008383958_1	181	WTAAFSIRYGNLYYNPFHMLSIAFLYGSA	VLFAMHGATVLAMSHYGGDREVEQIVDRGTA
WP_017667956_1	180	WT <mark>AAFSIRYGNLFYNPFH</mark> ALSIAFLYGST	LLFAMHGATILAVGRYGGEREIEQITDRGTA
AGY14263_1	181	WTNNFSLVHGNLFYNPFHGLSIAFLYGSA	LLFAMHGATILAVSRFGGDRELEQIAD
AEM00431_1	180	WTNLFSITHGNLFYNPFHALSIVFLYGSA	LFAMHGATILAVSRFGGEREIEQIVDRGTA
YP_006414082_1	181	WTAAFSIRYGNLYYNPFHMLSIAFLYGSA	LFAMHGATILSVSRFGGDREIDQIVDRGTA
CAQ16985_1	181	WTNLFSITYGNLFYNPFHALSIVFLYGSA	LFAMHGATILAVSRFGGEREIEQIVDRGTA
YP_003444494_1	181	WT <mark>AAFSIRYGNLYYNPFH</mark> MLSIAFLYGS <mark>A</mark>	LFAMHGATILAVSRFGGDREIDQITDRGTA

* Accession nos. refer to numbers listed in NCBI's protein database (http://www.ncbi.nlm.nih.gov/protein)

** Residues highlighted in black are conserved in 50% or more organisms. Residues highlighted in gray are not conserved, but are similar to a residue that is conserved in the same column (black letters). Residues in white are not conserved, nor are they similar to a residue that is conserved in the same column. Red column: position analogous to (M)214 in *R. sphaeroides*. Green: valine at position analogous to (M)214 in *R. sphaeroides*. blue: methionine at position analogous to (M)214 in *R. sphaeroides*.

APPENDIX 5: Light minus dark difference spectra of wild type and (M)L214G RCs before and after incubation with ubiquinone-10.



Absorbance spectra were not normalized, however the RC samples were kept at 42 μ M concentration. Legend: WT, wild type RC; WT + Q, wild type RC after incubation with 1/10 volume of saturated ubiquinone-10 in ethanol; (M)L214G, (M)L214G mutant; (M)L214G + Q, (M)L214G mutant after incubation with 1/10 volume of saturated ubiquinone-10 in ethanol.