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Light-Harvesting, Redox Control, and Biomimicry of the Photosynthetic Antennas of Green Sulfur Bacteria

Gregory Scott Orf
Washington University in St. Louis

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WASHINGTON UNIVERSITY IN ST. LOUIS

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Light-Harvesting, Redox Control, and Biomimicry of the Photosynthetic Antennas of Green Sulfur Bacteria
by
Gregory Scott Orf

A dissertation presented to the Graduate School of Arts & Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

May 2015
St. Louis, Missouri
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Greg Orf

Washington University in St. Louis

May 2015
This dissertation, and the work comprising it, is personally dedicated six-fold:

To my late father, for teaching me pride
To my mother, for teaching me compassion
To my sister, for teaching me humor
To my brother, for teaching me humility
And to my wife and son, for teaching me what life is really all about

--------

The development of a scientist’s worldview and ethical framework is essential to how he or she approaches the problems facing today’s world. I have modeled my own after the words of one of the greatest physicists the world has even seen, in which his sunny optimism shines forth:

“How wonderful that we have met with a paradox.
Now we have some hope of making progress.”

- Dr. Niels Bohr

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Abstract of the Dissertation

Light-Harvesting, Redox Control, and Biomimicry of the Photosynthetic Antennas of Green Sulfur Bacteria

by

Gregory Scott Orf

Doctor of Philosophy in Chemistry

Washington University in St. Louis, 2015

Professor Robert Blankenship, Chair

The green sulfur bacteria (class Chlorobea) are obligately anaerobic photoautotrophic prokaryotes. Members of this bacterial class are found in anoxic, sulfur- or iron-rich environments such as hydrothermal vents and hot springs. These bacteria are champions of low-light adaptation, with some species being able to thrive photosynthetically on less than eight photons hr$^{-1}$ per pigment. We have studied the two photosynthetic antenna complexes from these organisms that are responsible for light capture: the chlorosome and the Fenna-Matthews-Olson (FMO) protein.

In regards to the chlorosome, we studied, using steady-state and time-resolved spectroscopic methods, the effect of incorporation of an expected, but as-yet-undiscovered-in-nature, light-harvesting pigment into the complex, Bacteriochlorophyll (BChl)$f$. We show that BChl$f$ is a fully functional pigment in chlorosomes, although it transfers energy less efficiently to the next energy acceptors in the system. Additionally, we have pioneered methods to build self-assembling biohybrid chlorosome analogs that incorporate natural pigment and synthetic polymers that mimics the natural chlorosome structure. These biohybrid constructs look and act like natural chlorosomes, although their design offers flexibility of pigment choice not found in nature.
In regards to the FMO protein, we studied the effect of excitation intensity on the singlet and triplet excited states of the BChl \( a \) molecules in the protein using ultrafast spectroscopy. We show that the system undergoes intersystem crossing into the triplet state after \( \sim 25\% \) of excitations, and the lifetime of the triplet state is between 10-100 \( \mu s \). We also, using electrochemistry and mass spectrometry, identified the source of the FMO protein’s modulation of efficiency with respect to redox condition. One or both of the redox-sensitive cysteine residues in the protein, situated near BChl \( a \) #’s 2 and 3 modulate their redox state between free thiol and thyl radical form to quench BChl excitations, probably via an electron transfer/ultrafast recombination mechanism. These results together help us to understand the molecular mechanisms of photosynthetic and environmental robustness in these bacteria more completely.
Chapter 1: The Antenna Complexes of Green Sulfur Bacteria

This introductory chapter is designed to give the reader a brief introduction to the photosynthetic system of green sulfur bacteria before delving into the research projects outlined in Chapters 2-5. This chapter is partially adapted from the previously peer-reviewed and published first-authored review paper:


1.1 Introduction

Photosynthesis, the metabolic process that converts light energy into chemical energy, is accomplished via complexes containing pigments. Reaction center pigment-containing proteins use light energy to move electrons between molecules against their redox potential. Reaction centers are membrane proteins and in many cases have low absorption cross-sections. Large light-harvesting antenna complexes associate with reaction centers to increase the absorption cross-section while also regulating the flow of absorbed energy [1,2]. There are a myriad of light-harvesting pigments and antenna structures absorbing across the solar spectrum, all tuned to maximize their particular organism’s metabolic effectiveness in their own photic environment (Fig. 1.1).
Figure 1.1: The solar spectrum above the Earth’s atmosphere and at the Earth’s surface (black and grey lines, respectively, from the ASTM G-173 reference: http://rredc.nrel.gov/solar/spectra/am1.5/astmg173/astmg173.html), presented with the spectrum of a hypothetical 5778 K black body radiator loosely approximating the solar spectrum at Earth’s orbital radius, as well as the absorption spectra of all known natural bacteriochlorophylls and chlorophylls. The accessory pigments, carotenoids and bilins, absorb in the 450-700 nm range.

Green sulfur bacteria (class Chlorobea) and the phototrophic Acidobacterium Chloracidobacterium (Cab.) thermophilum, contain a main light-harvesting antenna complex called the chlorosome and a secondary antenna complex called the Fenna-Matthews-Olsen (FMO) protein. The filamentous anoxygenic phototrophs (phylum Chloroflexi) also contain chlorosomes, but lack the FMO protein [3–7]. The green sulfur bacteria photosynthesize
especially efficiently at the extremes of low photon flux; some species are adept at photosynthesizing using only the infrared (IR) radiation given off by deep sea thermal vents [8] or leftover solar radiation at the bottom of the photic zone in a stratified lake [9,10]. In these extreme cases, population doubling times may be on the order of years.

To achieve this excellent low-light efficiency, the light-harvesting complex must not waste any incident energy through competing relaxation or quenching processes. The chlorosome provides for efficient light absorption via its unique structure composed of an ellipsoid lipid monolayer sac encompassing thousands of self-assembled bacteriochlorophyll (BChl) c, d, e, or f pigments, as shown in Fig. 1.2. Unlike all other known light-harvesting complexes, which organize their pigments through pigment-protein interactions, chlorosome pigments self-assemble through ordered, long-range, pigment-pigment interactions independent of protein influence or stoichiometry [3,11–14]. This self-assembly process redshifts the absorption maximum of the BChl molecules into the near-IR range. In addition to the organized pigment oligomers, the interior of the chlorosome contains carotenoid and quinone molecules, which aid in light absorption and protection against reactive oxygen species (ROS) production [3]. The FMO complex acts as an energy transfer bridge, using BChl a molecules to transfer energy with high quantum efficiency from the chlorosome to the reaction center.
Figure 1.2 Schematic models of the light-harvesting systems of the three bacterial groups that contain chlorosome complexes: (A) a model of the system from the *Chlorobi* and *Cab. thermophilum*, and (B) a model of the system from the *Chloroflexi*. 
The history of chlorosome research is fascinating. The novel idea of self-assembled, non-random aggregation of BChl oligomers in chlorosomes was first suggested by A.A. Krasnovsky and co-workers beginning in the 1950’s through experiments simulating the *in vivo* BChl absorption using *in vitro* solid BChl films [15]. IR spectroscopy importantly revealed the coordination partners in each BChl molecule [16]. Research then moved to studying BChl aggregates in non-polar solvents in the late 1980’s and to aggregates in aqueous solvents in the early 1990’s, further revealing nuances in the oligomer structure and the requirements for organization [13,17–19]. This work with pigment organization eventually led to attempts to describe the functions of the various chlorosome proteins.

Various small proteins are found interspersed in the lipid monolayer sac of the chlorosome. Many of these proteins have obscure or unknown functions. The most important of these is the CsmA protein, which binds BChl *a* and oligomerizes to form a paracrystalline “baseplate” on a single face of the chlorosome. This baseplate serves as the intermediary in energy transfer between the self-assembled BChl *c/d/e/f* and the next energy acceptor in the system. As such, the CsmA baseplate offers the only pathway for energy to leave the chlorosome and provides directionality to the energy transfer. In *Chlorobea* and *Chloracidobacterium*, the next energy acceptor is the FMO protein. In *Chloroflexi*, the acceptor is the membrane-embedded B808-866 antenna complex. The FMO protein, or B808-866 protein, then transfers energy to the reaction centers [3,20].

An important feature of the BChl self-assemblies of the chlorosome is their long-range excitonic coupling. This excitonic coupling allows for extremely rapid energy transfer to the CsmA-BChl *a* baseplate with high quantum yield. This feature, combined with their size scale (all dimensions vary between 10-200 nm) and ROS self-protection abilities, has obvious
implications for nanomaterials science, especially for the development of efficient solar-capture devices [21].

The FMO protein was the first chlorophyll-containing protein to have its three-dimensional structure solved via X-ray crystallography [7]. It is a homotrimeric complex wherein each monomer internally binds seven BChl \(\alpha\) pigments, with an eighth pigment binding at the cleft between each monomer, adding up to 24 BChl \(\alpha\) molecules in an intact complex [22,23]. Because its structure is well-established and there is a wealth of optical data collected from it, the FMO complex has served as a test subject for many new techniques in photosynthesis, among which are two-dimensional and third-order electronic spectroscopies, mass spectrometric footprinting, and \textit{in silico} energy transfer simulations [24,25].

Recently, the FMO protein has generated renewed interest from the scientific community owing to the observation of quantum coherence after photon absorption by its pigments. Long-lived quantum coherence implies wave-like energy transfer character in which interferences among coherences lead to the system exceeding the classical limitations of energy transfer rate and efficiency [25–28]. This discovery led to a fundamental change in the way photosynthesis researchers view energy transfer dynamics. Additional evidence continues to mount that photosynthetic energy transport strikes a middle ground between purely coherent energy transport (quantum coherence) and purely incoherent energy transport (i.e., the FRET, or Förster Resonant Energy Transfer model, which assumes an incoherent protein bath environment) [25].

In this chapter, important aspects of the green sulfur bacterial antenna systems are discussed, including pigments, structure, spectroscopic properties, photosynthetic regulation, and unanswered questions. These topics offer an opportunity to study the physical properties of
pigments, metabolic evolution, horizontal gene transfer events, and novel methods that utilize the principles of nature to produce renewable energy for human use.

1.2 Photosynthetic Bacteriochlorophylls in Green Sulfur Bacteria

1.2.1 BCHls c, d, and e

The unique bacteriochlorophylls found naturally only in chlorosome-containing bacteria, BCHls c, d, and e, are collectively called the “Chlorobium chlorophylls.” These pigments, shown in Fig. 1.3, share a common basic structure but differ specifically at their C-7 and C-20 substituents. Variation at these two positions significantly alters their spectral properties. BCHls c and d have a methyl group at the C-7 position, whereas BCHl e contains a formyl group at this position. BCHls c and e contain a C-20 methyl group, which BCHl d lacks. This is the only group of naturally-occurring chlorophylls or bacteriochlorophylls to contain a C-3\textsuperscript{1} hydroxy group, lack a C-13\textsuperscript{2} methoxycarbonyl group, and variably alkylate their C-8 and C-12 positions [29]. These key differences allow for the unique self-assembly of these pigments, discussed in depth later.

In general, each organism will only contain one type of the “Chlorobium chlorophylls,” although exceptions are known [30,31]. The biosynthesis route for BCHls c, d, and e has been mostly elucidated following the development of a tractable genetic system in the early 2000’s by Donald Bryant and co-workers in the BCHl c-containing model green sulfur bacterium Chlorobaculum tepidum [32].
Fig. 1.3: (A) The chemical structure of the “Chlorobium chlorophylls” antenna pigment group. This particular compound contains a farnasyl tail, although phytol, geranylgeraniol, and non-branching long-chain alcohols can substitute, especially in the Chloroflexi. (B) The chemical structure of BChl $a$, which usually contains a phylly tail.

Through systematic inactivation of genes and analysis of pigment products, both new and functionally redundant steps were identified. Chlorophyllide (Chlide) $a$ is now believed to be the
Chapter 1

last common precursor between the “Chlorobium chlorophylls” group, BChl $a$, and Chl $a$; all of which are found in green photosynthetic bacteria [32]. Mutants unable to produce the $bciC$ gene product are unable to produce pigments without the C-13$^2$ methoxycarbonyl, thus identifying the first committed step from Chlide $a$ to eventual BChl $c/d/e$, although the enzyme’s mechanism is unknown [33]. The alkylations at the C-8, C-12, and C-20 positions that occur in the final pigment products are biologically difficult reactions as these are inactivated carbon centers. Because the cells avoid $O_2$, reactions normally handled by oxidases in oxygen-tolerant organisms must be done via radical mechanisms, notably radical S-adenosyl-methionine-dependent (radical SAM) reactions [34]. Searching the genomes of the Chlorobea and Chloroflexi for radical SAM-like enzymes identified the $bchQ$, $bchR$, and $bchU$ genes. The $bchQ$ and $bchR$ genes encode for the methyltransferases that variably alkylate the C-8 and C-12 positions. These alkylations do not change the spectral characteristics of individual pigments but play a role in the structural and spectral characteristics of the oligomeric structures and in light-adaptation [35]. The $bchU$ gene encodes for the C-20 methyltransferase enzyme. This step only occurs in BChl $c$- and BChl $e$-containing organisms, and was shown that some BChl $d$-containing organisms contain a version of the gene inactivated by a frameshift mutation [36]. The final step in the biosynthesis pathway is the addition of the hydrocarbon tail by bacteriochlorophyll $c/d/e$ synthase (BchK). Mutants in $bchK$ unable to synthesize bacteriochlorophyll $c$ synthase produce chlorosomes that only contain carotenoids (colloquially called “carotenosomes”) [37].

There are still gaps in knowledge pertaining to pigment biosynthesis, specifically the enzyme (or set of enzymes) that adds the formyl group to the C-7 position in BChl $e$. A single enzyme with this function is found in plants and certain cyanobacteria: the chlorophyll $a$ oxygenase (CAO) enzyme that converts Chl $a$ to Chl $b$. However, the CAO enzyme must be a
case of convergent evolution because it utilizes atmospheric $O_2$ as a substrate, a substrate unavailable to green bacteria, as discussed above [38]. Loss or gain of function in the C-20 methyltransferase enzyme and “C-7 formylase” enzyme are the sole reasons for the appearance of BChl c, d, and e in otherwise highly similar organisms. The table in Fig. 1.3 illustrates these differences. Very recently, a candidate gene in the BChl e-producing *Chlorobaculum limnaeum*, *bciD*, was inactivated by natural transformation, resulting in a mutant that produced only BChl c [39]. An analysis of the amino acid sequence shows that enzyme putatively belongs to the radical SAM enzyme family, although the exact mechanism of enzymatic action is unknown [39]. It is also currently unknown if this is the only enzyme involved in the C-7 transformation step.

### 1.2.2 BChl f

In 1975, a placeholder name was given for the last logical combination of C-7 and C-20 position substituents in the “*Chlorobium* chlorophylls” group: BChl $f$ [40]. This pigment is the 20-demethylated version of BChl e, and prior to the work described in Chapter 2, had only been synthesized from Chl $b$ and characterized *in vitro* [41]. It has still not been found in the wild, but only in laboratory-derived *bchU* null mutants of the BChl e-producing GSB *Cba. limnaeum*. Two such mutants were recently reported using two different gene inactivation pathways [42,43]. In both mutants, the BChl $e$ content of the chlorosomes is completely shifted to BChl $f$. The current hypothesis, which is shared and justified in Chapter 2, is that BChl $f$-containing organisms may exist in nature but are rare because they would require an anoxic niche devoid of competition from their BChl c-, d-, and e-containing counterparts, as well as Chl $a$- and $d$-containing organisms [44].
1.2.3 BChl $a$

BChl $a$ comprises about 1-2% of the total pigment content in the chlorosome, but constitutes all pigments in the FMO protein and nearly all pigments in the photosynthetic reaction center of GSB species. In the chlorosome, it is found as a component of the paracrystalline CsmA baseplate, which localizes on one face of the chlorosome and accepts energy from the self-assembled BChl $c$, $d$, $e$, or $f$, providing directionality for energy transfer [45–48]. From the common precursor Chlide $a$, there are only four additional steps necessary to synthesize BChl $a$: a 3-vinyl-BChlide hydratase (BchF), chlorophyllide $a$ reductase enzyme (BchX-BchY-BchZ), a 3-hydroxyethyl BChlide $a$ dehydrogenase (BchC), and the tail-appealing bacteriochlorophyll $a$ synthase (BchG). Some of these enzymes are homologous to those from the analogous pathway in purple bacteria [32,49,50].

1.3 The Organization of the Chlorosome

1.3.1 BChl Oligomeric Structure

Chlorosomes generally are 100-200 nm in length, with width and depth dimensions between 10-60 nm [6,51,52]. There are, depending on species, between 50,000 and 250,000 BChl pigments per chlorosome [53–55]. The nature of the chlorosome’s exterior lipid monolayer encourages the self-assembly of its interior pigments. All of the lipids arrange with tail groups pointed into the interior of the chlorosome, generating a highly hydrophobic interior. There is a need to pack the pigments in a way to maximize polar/polar and non-polar/non-polar contacts [56]. The nature of the short- and long-range structural features of these BChl oligomers has been a contentious point for some time. Cryo-EM has produced detailed images of the chlorosome interior, leading to competing theories of pigment assembly “lamellae,” “spirals,”
and “nanotubes” [57–61]. Unfortunately, X-ray crystallography is not suitable for chlorosome study owing to natural variation between and within each chlorosome [58].

The key for short-range interaction between two BChls in the system was found, first through IR experiments, to be a coordination bond between the C-3\(^1\) hydroxy group and the central Mg\(^{2+}\) above or below the plane of the chlorin ring [16] (see Fig. 1.4). The C-13\(^1\) keto group may also play a role in coordination [16–18]. The lack of a C-13\(^2\) methoxycarbonyl group also removes a large source of steric hindrance that would otherwise disrupt this coordination.

To reduce the problem of structural heterogeneity in chlorosomes caused by the variable alkylations at the C-8 and C-12 positions, and study the BChl oligomer structure, mutants were produced such as \textit{Cba. tepidum ΔbchQRU} and Δ\textit{bchQR}, which produce homogeneous BChl \textit{d} or \textit{c} pigment., respectively. Fourier transform cryogenic electron microscopy (FT-cryo-EM) and \textsuperscript{13}C-\textsuperscript{13}C, \textsuperscript{1}H-\textsuperscript{13}C solid-state NMR of the homogenous system produced detailed structural information [35,62]. BChl stacks are spaced about 2 nm apart from each other and have an axial repeat varying between 0.8-1.2 nm. Additionally, the structure of individual BChl dimers was shown to be consistent with a syn-anti model [62]. Further complicating the overall structure, the syn-anti structures can introduce heterogeneity in the bulk aggregates by joining syn- and anti-coordinated domains with helical transition regions, shown in Fig. 1.4 [63]. Overall, mutant and wild-type chlorosomes show different helical packing organization, all based on syn-anti dimers arranging themselves in either a mixed or one type of syn- or anti-ligation.
Fig. 1.4 Schematic models of a section of a chlorosome BChl tube from the (A) bchQR mutant and (B) WT. Extended domains from (A) can be joined by random syn-anti interfaces to introduce heterogeneity in an otherwise highly regular scaffold, as depicted in (C). The long arrows indicate the direction of the chlorosome long-axis. A: Anti-coordinated BChl molecules; S: Syn-coordinated BChl c molecules. Figure reprinted with permission from Ref. [63]. Copyright 2012, American Chemical Society.

Altogether, these data paint a complex picture of the oligomer structure in wild type chlorosomes and illustrate the importance of the C-3 1-hydroxyethyl group, the C-8/C-12
variable alkylations, and the C-20 methylation. By mixing variable methylations into the building block BChls of the stacks, a tubular structure can be built that is sufficiently heterogeneous to confer increased absorption bandwidth (a property known as “inhomogeneous broadening”), but is sufficiently homogeneous to be stable for a long period of time. Organisms incorporating variable methylations outperform all pigment mutants in doubling time in all light intensities [35]. It is of note that the pigment aggregation distance constraints seen in chlorosomes from the FAP Cfx. aurantiacus are larger than those from the GSB species, likely due to the longer length of the BChl c tails in this species [61].

It is also important to point out that regardless of the supramolecular stack direction, the hydrogen-bonding network, formed by a C-13\textsuperscript{1} carbonyl interacting with a C-3\textsuperscript{1} hydroxy hydrogen, aligns the pigment transition dipoles in the direction of the oligomer stacking such that exciton delocalization can move up or down the oligomer. This additive extended dipole moment is, therefore, a source of the rapid and efficient energy transfer in the system [63–66]. Thus, we can thus conclude in general terms, that the wide variety of internal chlorosome structures seen in beautiful published EM images [58,63] are the results of very slight variations at the molecular level – sometimes only by one carbon per molecule! – in an otherwise well-ordered syn-anti dimer-based system. The structures appear regular on the level of tens of nanometers but begin to diverge in structure over the entire 100+ nm length of the chlorosome.

1.3.2 Other Internal Chlorosome Components

Other than self-assembled BChl, there are other components inside the chlorosome that aid in absorption, triplet state quenching, and redox control. Carotenoids (Car) are one of these important inclusions, representing up to 10% of the pigments in each chlorosome [5]. Carotenoids can serve either as an energy donor to the surrounding BChl molecules or can serve
as an energy acceptor for BChl molecules in a triplet excited state, $^3$BChl*. $^3$BChl* is particularly dangerous for cells exposed to $O_2$, as it can transfer energy to ground state $^3$O$_2$ via a collisional electron exchange mechanism, producing $^1$O$_2^*$, a long-lived ROS. Carotenoids can accept energy from $^3$BChl*, forming $^3$Car*, and can safely dissipate that energy primarily through internal conversion [67]. On the other hand, useful carotenoid-BChl energy transfer proceeds from the singlet $S_2$ excited state of carotenoids, or the optically-forbidden $S_1$ state via $S_2 \rightarrow S_1$ internal conversion, to a singlet $Q$ excited state of the BChl [68]. Carotenoids also influence the self-assembly of BChl oligomers by competing for BChl-BChl hydrogen and coordination bonding [69]. Chlorosomes from GSB species generally contain chlorobactene (or derivatives), $\gamma$-carotene, or isorenieratene, whereas chlorosomes from Chloroflexi contain various $\beta$- and $\gamma$-carotenes [70–72].

Quinones are also interspersed throughout the interiors of chlorosomes, generally amounting to a concentration of about 0.1 mol per 1.0 mol of BChl. Quinones function as excitation quenchers in the presence of oxygen to slow down the rate of photosynthesis [73]. Green sulfur bacteria in particular contain susceptible Fe-S clusters in their reaction centers, which, in the presence of oxygen and energy donated from the chlorosome, can produce superoxide ($O_2^-$), another ROS. The presence of quinones is therefore a mechanism for cell survival in oxidizing conditions [3,74]. Under oxidizing conditions, the quinones are likely oxidized into photoactive species that quench excitation energy from neighboring BChl molecules, most likely by electron transfer followed by ultrafast recombination. Chlorosomes from the Chlorobi mostly contain chlorobiumquinone ($1'$-oxomenaquinone-7) with a small amount of underivitized menaquinone, whereas chlorosomes from Chloroflexi only contain menaquinone [73]. The chlorosomes from Cab. thermophilum contain a reduced menaquinone
derivative; this derivative contains a reduction in one of its isoprenoid units, the role of which is unknown [75].

1.3.3 Chlorosome-Localized Proteins and the CsmA Baseplate

The lipid monolayer surrounding the chlorosome pigments is interspersed with surface-exposed proteins. These proteins have been best characterized in the chlorosomes from the *Chlorobi*. Chlorosomes from the *Chlorobi* can contain up to eleven different protein types: CsmA, CsmB, CsmC, CsmD, CsmE, CsmF, CsmH, CsmI, CsmJ, CsmK, and CsmX. Only CsmA, CsmC, and CsmX are universally encoded in all of the *Chlorobi* and only CsmA appears in all chlorosome-bearing taxa (Fig. 1.5A) [76]. Other unique chlorosome proteins are also found in the *Chloroflexi* (CsmM, CsmN, CsmP, and CsmY) and *Cab. thermophilum* (CsmR, CsmS, CsmT, CsmU, CsmV) [77–79].

In *Cba. tepidum*, the proteins are divided into four distinct structural motifs: CsmA/E, CsmB/F, CsmC/D, and CsmI/J/X. The relation within motifs is likely due to gene duplication events that added functional redundancy. The CsmB/F and C/D families seem to affect pigment synthesis levels and assembly, affecting the size of the chlorosome [76]. The CsmI/J/X family appears to bind [2Fe-2S] clusters, participating in the aforementioned redox control of the chlorosomes through ferredoxin-like activity [80–82].
Fig. 1.5 (A) An amino-acid sequence alignment of the CsmA baseplate protein from representatives of the three bacterial groups containing chlorosomes, generated using Clustal Omega. *Chp. = Chloroherpeton*, a GSB. Green letters: conserved residues composing the BChl \( a \) binding site; Blue letters: conserved INRNAY sequence; the red “|” denotes the site of post-translational cleavage, although we hypothesize this placement in *Cab. thermophilum* based on homology. (B) From left to right: top, side, and bottom views of the FMO protein from *Cba. tepidum* (PDB: 3ENI), with regions packing closely to the CsmA baseplate highlighted in red. Part B reproduced with permission from Ref. [83]. Copyright 2012, American Chemical Society.

The only chlorosome-localized protein absolutely essential for cell viability is CsmA [12]. As mentioned before, it binds BChl \( a \) (and likely carotenoids) and arranges many copies of itself into a two-dimensional paracrystalline structure [45–48,79,84]. The structure of a single CsmA monomer from *Cba. tepidum* was determined by solution NMR to contain two alpha helices: the N-terminal helix (residues 6-36) embeds in the chlorosome lipids and the C-terminal
helix (residues 41-49) associates with the FMO protein [85]. The structures of the paracrystalline arrays are likely built by repeating dimer units driven to associate by hydrophobic interactions between the N-terminal alpha helices of two adjoining copies of the protein [86–88]. Binding of BChl $a$ to CsmA redshifts the BChl $a$ Qy absorption maximum by $\sim$30 nm [89]. Circular dichroism spectroscopy also shows that the BChl $a$ pigments in the baseplate interact weakly in an exciton-coupled manner, allowing for limited excitation delocalization [48,89].

It appears likely, from clues from both genetic and computation studies, that the lone, conserved histidine residue in CsmA binds the BChl $a$ pigment [88]. It is not known exactly how the CsmA proteins interact with the FMO or B808-866 complex, although at least in $Cba. tepidum$, a broad consensus amino acid sequence near the C-terminus of the mature protein (INRNAY) is involved [90]. Hydrogen-deuterium exchange mass spectrometry experiments revealed the amino acids in the $Cba. tepidum$ FMO protein that pack closely with CsmA (residues 123-123, 140-149, 150-162, 191-208, and 224-232; Fig. 1.5B) [83]. However, the details of the interaction, from both structural and energy-transfer standpoints, are still unclear.

1.4 Energy Transfer Properties of Chlorosomes

The general pathways for energy transfer within chlorosomes described thus far are summarized for clarity within a Jablonski diagram (Fig. 1.6).
Fig. 1.6 Jablonski diagram on an arbitrary energy axis depicting the general modes of excitation energy transfer among chlorosome components. Each excited state energy level except for the Car and O$_2$ S$_1$ states and the oxidized quinone excited state can be directly accessed via single photon absorption under physiological conditions. Red lines: energy transfer events beneficial for photosynthetic productivity; black lines: internal conversion; blue lines: intersystem crossing; green lines: phosphorescence or internal conversion; purple lines: BChl triplet quenching pathways.

In monomeric form, the “Chlorobium chlorophylls” have absorption spectra similar to Chl $a$, with Q$_y$ maxima between 645 to 670 nm (Fig. 1.7A). Upon oligomerization in the chlorosome, the pigment absorption bands widen, and the Q$_y$ red-shifts by 60 to 80 nm (BChl c: 670 nm to ~750 nm; BChl d: 657 nm to ~730 nm; BChl e: 660 nm to ~720 nm; BChl f: 645 nm to ~705 nm; Fig. 1.7B) [41–43,91,92]. There is a structural relationship between the spectral
differences in the “*Chlorobium* chlorophyll” monomers: the addition of the formyl group at the C-7 position will blue-shift the absorption maximum of the $Q_y$ band by about 10 nm, while the addition of a methyl group at the C-20 position will redshift the absorption maximum of the $Q_y$ band by about 15 nm. It is also important to note that the monomeric pigments bearing C-7 formyl groups display a $Q_y$ absorption band that is decreased in intensity by ~50% relative to their C-7 methyl counterparts. The heterogeneity and coupling of the BCHl oligomers in the chlorosome red-shifts and inhomogeneously broadens the absorption peaks in chlorosome: the addition of the formyl group at the C-7 position will blue-shift the absorption maximum of the $Q_y$ band by about 30 nm, while the addition of a methyl group at the C-20 position will red-shift the absorption maximum of the $Q_y$ band still by about 15 nm.
Fig. 1.7 (A) Absorption spectra of the “Chlorobium chlorophylls” group member pigments dissolved in pyridine (monomeric form, central Mg atom hexa-coordinated), (B) Absorption spectra of native chlorosomes representative of each of those four pigments, and (C) Gaussian simulations of the spectral overlap between BChl oligomer donors and the CsmA-BChl a baseplate acceptor within chlorosomes.
The changes brought about by the C-20 methylation are explained through a small puckering of the BChl ring [93,94]. The changes brought about by the C-7 formylation are more pronounced and may be explained by the larger electron-withdrawing effects of the -CHO group [95]. Singlet excited state lifetimes of the monomeric pigments are only slightly changed via C-20 methylation (~7% increase, due to methylation), but the addition of the C-7 formyl group decreases this value by over 50% [92]. Similar trends are seen in the fluorescence quantum yield values (see Chapter 2) [44].

Many studies have investigated the structural and excitonic coupling of the BChl oligomers and how they affect the chlorosome absorption spectrum. While the coupling allows for a broadened and red-shifted absorption spectrum, it also allows for very efficient and very fast excitation energy transfer (EET) out of the system. Two-color pump-probe and two-dimensional electronic spectroscopy experiments demonstrate intra-aggregate BChl-BChl EET in less than 100 fs for the FAP Cfx. aurantiacus and less than 270 fs for GSB’s Cba. tepidum, Prosthecochloris (Pst.) aestuarii, and Cba. phaeobacterioides (now called Cba. limnaeum) [96–98]. Calculations based on pump-probe experimental data predict that, on average, about 24 exciton-coupled pigments at a time can participate in these fast EET times [99]. Carotenoid (Car)-BChl EET can occur in less than 500 fs and proceeds from the S_2 excited state of the Car [68,69,100]. Recent MD and density function theory (DFT) calculations using recent oligomer structural models reveal that excitations in the BChl oligomers diffuse over portions of the aggregate elements in less than 200 fs, similar to what is shown for the FMO protein [98,101]. In Cfx. aurantiacus, BChl c to baseplate BChl a EET occurs in less than 16 ps, [102,103] but not less than 6.6 ps [97]. In Pst. aestuarii and Cba. tepidum, these same transfer times can occur in less than 12 ps [97]. The peripheral BChl to baseplate transfer rates and efficiencies vary from
species to species, especially when BChl c is replaced with the blue-shifted BChl d, e, or f.

Because the baseplate absorption varies little between chlorosome types, decreases in spectral overlap between the oligomer BChl fluorescence and the baseplate absorption explain the longer energy transfer times and lower energy transfer efficiency rates between the two species [44,104]. The rate and quantum yield of energy transfer specifically from the baseplate to the FMO protein or the baseplate to the B808-866 complex are unknown, although we can speculate these decay times are under 200 ps.

Circular dichroism (CD) has also been used to investigate the structure of the BChl oligomers based on how the structures affect circularly polarized light [105]. The shape of the chlorosome CD spectra in the near-IR regions is generally S-shaped, indicative of excitonic interactions between pigments. However, reports of the intensity of the (+) and (−) lobes, as well as the order in which they appear, are widely variable across the literature. This is believed to stem from the structural heterogeneity of the BChl aggregates, especially the angle between the assemblies and the chlorosome horizontal axis and the assembly lengths [64,106]. Therefore, CD spectra can vary not only for chlorosomes from cells grown in different light conditions, but even from seemingly identical cell preparations.

### 1.5 The Organization of the FMO Protein

As briefly described above, the FMO protein is a soluble homotrimeric protein that binds seven BChl a pigments inside each monomer and an eighth BChl a at the interface between each monomer, totaling 24 BChl a pigments in an intact complex. The secondary structure of each monomer is primarily β-sheet, with α-helices appearing only at the periphery, near where the monomers associate with each other [7,23,107–110]. Because of the three-dimensional shape of the protein, the complex is colloquially called the “taco-shell” protein, in which the protein forms
the shell of a taco, with the pigments nesting inside the shell, acting as “filling” [108]. Viewed from above (perpendicular to the plane of the cell membrane), the intact trimeric complex is approximately 8 nm in diameter, while viewed from the side (parallel to the plane of the cell membrane), the intact complex is just over 5 nm tall [111]. The monomers interact with each other through many strong polar interactions and salt bridges between amino acids. Because these interactions are strong, the FMO protein has never been separated into stable free monomers. The three-dimensional structure of the protein is shown in Fig. 1.8.

The BChl \(\alpha\) molecules inside the complex (BChls #1-7) are coordinated at the central Mg ion by various ligands. For all of these BChls, the Mg ion is penta-coordinate, with four coordination partners being the chlorin ring nitrogens, and the fifth ligand coordinating above or below the plane of the chlorin ring. In BChls #1, 3, 4, 6, and 7, the fifth ligand is the imidazole nitrogen of different histidine residues. For BChl #5, the fifth ligand is the backbone carbonyl oxygen of a leucine residue. For BChl #2, the fifth ligand is a water molecule that is additionally hydrogen-bonded to the hydroxyl side group of a serine residue [24,108]. No covalent interactions have ever been resolved between the pigments and protein or between the pigments themselves. Intra-monomer, the chlorin rings of neighboring BChl \(\alpha\) ligands are separated by 4 - 11 Å, whereas inter-monomer, the corresponding distances are never less than 20 Å. These distance constraints suggest that energy transfer between two pigments in two different monomers is highly unfavorable, although there is some debate in this area [112].
Figure 1.8: The three-dimensional structure of the FMO protein from *Pst. aestuarii* strain 2K derived from Protein Data Bank entry 3EOJ and modelled with PyMol version 1.7. The protein-solvent contact surface is shown in transparent blue. (A) The structure from above (the perspective of the chlorosome, perpendicular to the plane of the cell membrane) showing the C$_3$ symmetry of the monomers, (B) the structure from the side (parallel to the plane of the cell membrane), and (C) a pigment-emphasized structure from the side, with each BChl $a$ labeled by number according to established convention. In (C) only, the BChl $a$ phytol tails are omitted for clarity.

The existence of the eighth BChl $a$ pigment at the interfaces between each monomer has been a contentious point for some time. In some of the original experiments performed on the complex, a preliminary BChl $a$ count of 21-23 was calculated. But, once the trimeric nature of the complex was established and the first crystal structures produced, the BChl $a$ count was
amended to 21 [7]. Beginning in 2004, refinements of the FMO crystal structure from both Cba. tepidum and P. aestuarii suggested that extra, unaccounted electron density near the monomer interfaces could be indicative of an extra BChl a pigment [113]. Further refinements of the Cba. tepidum structure and a new diffraction dataset for the Pst. aestuarii structure showed that an eighth pigment was indeed present in both structures, although the local protein environment and coordination partners in each protein were different. This difference in coordination environment accounts for the differences seen in the optical spectra between the protein from the two species. In Cba. tepidum, the BChl a #8 central Mg ion is penta-coordinate like all of the other pigments, but in Pst. aestuarii, three sequence differences (T165F, A168S, insertion of 1 amino acid at position 174) allow for an additional coordination to BChl #8 by the hydroxyl group of Ser168, making the central Mg ion hexa-coordinated [23].

Primary sequence alignments of various GSB species indicates that their FMO protein can fall generally into two classes: type I (including Pst. aestuarii) has a hexa-coordinated BChl a #8, while type II (including Cba. tepidum) has only a penta-coordinate BChl a #8. These structural changes manifest themselves in optical changes at low temperatures in the Qy region (see section 1.6) [23].

The occupancy level of the eighth pigment was also under debate until recently. Because of the difficulty in crystallographically capturing sufficient electron density for model-building, it was reasoned that purification of the protein somehow strips a sizeable population of BChl a #8 away, but in vivo, the occupancy of the pigment is near unity. Experiments using the newly developed mass spectrometry technique, native ESI-MS, demonstrated that the occupancy of the eighth BChl a varies depending on the harshness of purification conditions [22]. In fact, in most preparations of the protein, at least one of the BChl a #8 molecules can be lost. Interestingly,
during mass spectrometry, the phytol tail of BChl a #8 is lost by fragmentation. Crystallographic efforts also could not capture the electron density of the phytol tail. But, analytical HPLC on intact complexes shows that only intact BChl a is present in the complex. These results suggest that the tail of BChl a #8 is highly flexible and likely points out of the complex [22]. Mass spectrometry also confirmed the orientation of the FMO protein on the membrane through extensive chemical footprinting experiments; the BChl a #8 side of the complex is clearly situated near the chlorosome CsmA protein, whereas the BChl a #3 side binds to the reaction center [83,114]. These results together suggest that the phytol tail of BChl a #8 associates with the CsmA baseplate of the chlorosome or buries itself into the chlorosome lipid monolayer.

1.6 Energy Transfer Properties of the FMO Protein

The FMO protein maximally absorbs light at around 370 nm (Soret transition), 600 nm (Q_x transition), and 810 nm (Q_y transition), with the 810 nm transition being the strongest. The positioning of the 810 nm absorption band allows for efficient acceptance of energy from the CsmA-BChl a baseplate, which fluoresces maximally around 810 nm. The FMO protein itself maximally fluoresces around 820 nm, allowing for good energy transfer to the RC, which absorbs closer to 830 nm. When the complex is cooled to cryogenic temperature, the Q_y transition splits into three or four separate peaks, representing three or four excitonic bands, or groups of pigments close to each other in site energy. Representative absorption spectra are shown in Fig. 1.9. As mentioned in section 1.5, the coordination pattern of BChl a #8 varies between groups of organisms. The spectra in Fig. 1.9 are of the FMO protein from *Cba. tepidum* (type II FMO). In the 77 K spectra, the 809 nm absorption band is the strongest, followed closely by the 815 nm band. In the protein from *Pst. aestuarii* (type I FMO), the intensity order is switched so that the 815 nm band is the most intense. Therefore, something as simple as an extra
coordination ligand to a BChl has a pronounced effect on the optical character of the complex as a whole.

![Absorbance spectra of the FMO protein from Chlorobaculum tepidum](image)

**Figure 1.09:** Absorbance spectra of the FMO protein from *Chlorobaculum tepidum* (a type II FMO) at room temperature (main panel) and at cryogenic temperature (inset).

The site energies and contribution of each individual pigment to the overall spectrum of the protein is a complex and hotly debated issue. From a biochemical standpoint, arguably only the assignments of the highest and lowest energy sites in the complex are important; these assignments, combined with the three-dimensional structure, tells us about the orientation of the protein and how it interacts with the other photosynthetic complexes. From a more physical
standpoint, the site energy values are necessary for calculations of probable energy transfer pathways and exciton dynamics. These site energies are derived from calculations of the protein environment surrounding the pigments and the orientation of the pigment dipoles relative to one another, and are simultaneously fit to mimic various optical spectra already collected for the protein. Over time, the assignment of lowest site energy has changed. Once believed to be BChl a #7, BChl a #3 is now widely accepted to be lowest-energy pigment in the complex, with an energy situated between 820-822 nm [24,115]. It was postulated that the dipoles of the α-helices in the complex impart strong partial charges near BChl a #3, which redshifts the site energy by almost 300 cm$^{-1}$ [116]. This site assignment agrees well with the mass spectrometry evidence showing that this pigment should be the exit pigment and is situated next to the reaction center [114].

Due to the close spacing of BChls (< 10 Å apart) in each monomer, there is significant electronic coupling between pigments. This allows the electronic transitions in the protein to be delocalized over many pigments, which can be described in terms of excitons. To compute the exciton energy levels and describe their wavefunctions, the following electronic Hamiltonian operator, $\hat{H}_0$, accounting for $N$ coupled pigments is used,

$$\hat{H}_0 = \sum_{p \neq 1}^N E_{pp} |p\rangle\langle p| + \sum_{p \neq q}^N V_{pq} (|q\rangle\langle p| + |p\rangle\langle q|)$$  \hspace{1cm} \text{Eq. 1}$$

where $E_{pp}$ is the site energy for a non-interacting pigment $p$ that depends on the protein environment around the pigment, and $V_{pq}$ is the interaction energy between pigments $p$ and $q$. Evaluation of this Hamiltonian results in an $N \times N$ matrix containing site and interaction energies. Diagonalizing the $N \times N$ matrix yields the wavefunctions (eigenvectors) and expansion coefficients, $C$, for all $N$ excitons.
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The $q$-th exciton level is an excited state delocalized over all $N$ pigments, with the share of each pigment $p$ in forming that state given by the expression $C_{pq}^2$. The expansion coefficient allows for an evaluation of how much each pigment contributes to an observed absorption band in the complex and for a simulation of what pathways energy takes through the complex [24,117,118].

The confirmation of an eighth BChl $a$ pigment in the protein aided simulations of excitation energy flow through the FMO protein because the entrance pigment to the complex was now known. These simulations generally follow Redfield Theory, or modifications thereof, to model exciton relaxation pertubatively, providing excited state populations for each interacting pigment at various time periods after initial excitation [116,119]. Redfield Theory holds in situations where the pigment-pigment interactions outweigh pigment-protein interactions. An example of this treatment is shown in Fig. 1.10, which is reproduced from Ref. [115].
Figure 1.10: Flow of excitation energy through the pigments of the FMO protein from *in silico* simulation. At $t = 0$, it is assumed that the chlorosome baseplate transfers energy to BChl $a$ #8. The top panel shows the BChl bacteriochlorin rings colored according to their population at a given time after excitation (darker colors indicate higher population). The bottom panel quantitatively shows the occupation probabilities of individual pigments as excitons relax in the complex after initial excitation. Figure reprinted with permission from Ref. [115]. Copyright 2012, American Chemical Society.
Treatments such as this suggest that energy reaches BChl $a$ #3, the exit pigment, in high population in a matter of a few picoseconds. One study suggests that energy can reach BChl $a$ #3 through different branches, each with their own relaxation (equilibrium) rates [115]. The site energy gap between BChl $a$ #8 and #3 is about 400 cm$^{-1}$, which is about twice the thermal energy at physiological temperature ($\sim 210$ cm$^{-1}$). This an excellent tuning feature of the protein; BChl $a$ #8 (site energy $\sim 806$ nm) is poised to efficiently accept energy from the chlorosome, but once energy moves to BChl $a$ #3 (site energy $\sim 822$ nm), it is very unlikely to leak back into the chlorosome [115,120].

As mentioned in Section 1.1, the FMO displays characteristics of quantum coherence amongst its pigments. An extensive introduction to this topic will not be given because the relevance of coherence in physiological conditions on the efficiency of the protein is in a general state of disagreement among the community. Quantum coherence essentially means that energy traverses through the complex in a wave-like fashion, where the electronic states of the system are in a state of superposition [121]. This type of transfer starkly contrasts to semi-classical models like FRET, in which energy “hops” from chromophore to chromophore and only one electronic state can be occupied at a time. Coherent energy transfer allows for faster transfer rates than the semi-classical “hopping” model because the system can randomly walk through different excited states and sample different pathways simultaneously to find the most efficient path [28]. Coherence can be inferred by the measurement of quantum “beating” of chromophores by using two-dimensional Fourier transform electronic spectroscopy, in which excitation and emission energies are correlated as a function of delay time between excitation and emission events. These spectra are plotted as absorption frequency $vs.$ emission frequency, where diagonal cross-peaks indicate electronic coupling between chromophores [121].
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Theoretical models suggested that coherences among the pigments should dephase before 250 fs (within the same timescale of the first transfer events in FMO), but beating effects have been observed lasting longer than 1000 fs [26–28]. These effects can persist on the timescales relevant for complete energy transfer through the complex, but it is unclear whether or not the coherence actually provides an *in vivo* advantage for the protein. If quantum coherence does prove to be present among many kinds of antenna complexes across disparate taxa, then this suggests a distinct evolutionary advantage. Wavelike energy transfer would allow for especially efficient energy transport in systems where there are many local energy minima but no distinct “downhill” path towards the reaction center (e.g., the antennas of higher plants). In these systems, coherence would simplify the energy transport scheme by “course-graining” the energy landscape [25].

As a final point, like the chlorosome, the FMO protein is able to quench excited BChl states effectively when the surrounding environment moves from reducing to oxidizing redox potentials [122]. This redox-dependent quenching mechanism likely does not function in the same way that the mechanism in the chlorosome functions; no quinone molecules have ever been found bound to or within the complex. In reducing conditions, the pigments in the isolated FMO complex fluoresce with a major lifetime of about 2 ns. This lifetime shrinks to about 60 ps in oxidizing conditions, suggesting that a redox-modulated functional group inside the protein is able to quench excitations effectively. The circular dichroism spectrum of the protein also varies depending on redox condition, suggesting that the pigment orientations slightly change after encountering oxidizing conditions. It was hypothesized in the early 1990’s that this mechanism exists so that the rate of excitation quenching will outcompete energy transfer from FMO to the
RC in oxidizing conditions, effectively acting as a “volume knob” for photosynthesis [123]. A molecular mechanism for this process will be proposed in Chapter 5.

1.7 Unresolved Questions: Chlorosome Pigment Selection and FMO Redox Control

Gaps in our knowledge remain concerning green sulfur bacterial photosynthesis. As a general rule, green sulfur bacteria seem to favor chlorosomes that only contain one type of main pigment; BChl c, or d, or e, but not combinations of them at the same time. It would seem that the light-harvesting efficiency (in terms of wavelength range) would be increased by using multiple types of pigments in tandem within chlorosomes. A wider possible range for light absorption would aid these organisms in competition against other photosynthesizers. Is there a more fundamental reason why this strategy has not been adopted by green sulfur bacteria? The new availability of mutant GSB strains containing BChl f also poses the question of why species containing this pigment have not yet been found in the wild. Is there a physical or molecular reason why the ability to produce BChl f is not a desirable trait?

Regulation in photosynthetic light harvesting is very diverse across taxa. This field of study has grown in recent years. Light harvesting regulation in green sulfur bacteria is intimately tied to the redox potential and oxygen concentration of their growth condition. The chlorosomes are able to quench excitations by using quinones that are interspersed among the self-assembled BChl in their interiors. The FMO protein clearly displays redox-dependent regulation as well, although its molecular mechanism is unknown. How is the FMO protein able to mitigate triplet excited states effectively? Does this redox-dependent quenching mechanism represent a unique molecular mechanism in photosynthesis?
1.8 Goals of the Thesis

This thesis seeks to advance our understanding of light-harvesting efficacy and light-harvesting regulation in green sulfur bacterial photosynthesis. Advances in this area are instrumental to understanding irreducible aspects of photosynthesis such as pigment binding and excited state control. Next-generation solar cell technology will likely be influenced by the study of photosynthesis, with inspiration for design coming from the structure of the natural systems that have evolutionarily prevailed on Earth for the last two billion years.

Due to the recent availability of bacteria containing bacteriochlorophyll \( f \) for study, it is possible to study energy transfer and pigment assembly in chlorosomes more closely than before. Advances in mass spectrometry and ultrafast optical spectroscopy allow us to study proteins at an incredible level of detail at the atomic scale. The goals of this thesis are to:

1. Study bacteriochlorophyll \( f \) in chlorosomes and in organic solvents to determine its basic photophysical characteristics and efficiency with which it transfers energy,
2. Pursue new ways to mimic chlorosome pigment assembly \textit{in vitro}, including incorporating multiple types of pigments (including BChl \( f \)) into the same complex,
3. Study the dynamics of the triplet excited state of the FMO protein, and in particular how the protein protects itself against damage caused by triplet excited states, and
4. Study the molecular mechanism of redox-dependent excitation quenching in the FMO protein.

These goals will be accomplished through highly collaborative research and a multi-disciplinary approach. We will specifically combine time-resolved ultrafast optical spectroscopy and high-resolution mass spectrometry to leverage our laboratory’s strengths, as well as employ traditional analytical biochemistry and molecular biology to complement our findings.
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1.9 References


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Chapter 2: Production and Characterization of Bacteriochlorophyll $f$ in Chlorosomes

This chapter is adapted from the previously peer-reviewed and published first-authored manuscript:

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2.1 Introduction

As discussed in Chapter 1, chlorosome antenna complexes are essentially sacs filled with pigments, in which a lipid monolayer envelope interspersed with proteins surrounds self-assembled, light-harvesting bacteriochlorophylls (BChls). Single chlorosomes contain between 100,000 and 250,000 BChl $c$, $d$, or $e$ molecules, depending on species. The self-assembly of the BChl pigments is largely independent of protein influence, and several oligomeric structural patterns were identified [1–8]. Carotenoids and quinones, which aid in light absorption, triplet state quenching, and redox control, are also dispersed throughout the interior of the chlorosome. The self-assembled BChls transfer excitation energy to the CsmA baseplate, a paracrystalline BChl $a$-containing protein complex integrated into the lipid monolayer of the chlorosome. The baseplate serves as the interface to the FMO protein [1].

Bacteriochlorophylls $c$, $d$, and $e$ (which are actually chlorins, not bacteriochlorins, as evidenced by the oxidation of the C-7–C-8 bond in Ring B) share a common basic structure but
differ specifically at their C-7 and C-20 substituents, which significantly alter their spectral properties. BCHls c and d have a methyl group at the C-7 position, while BCHl e has a formyl group at this position. BCHls c and e contain a C-20 methyl group, which BCHl d lacks. There has long been a placeholder name for a fourth member of the “Chlorobium chlorophyll” group, BCHl f, even though it has not yet been observed naturally [9–11]. The presence of the C-7 formyl group and absence of the C-20 methyl group in BCHl f represent the last possible combination in the “Chlorobium chlorophyll” pigment family (see Fig. 1.03 in Chapter 1).

Through the development of a tractable genetic system in the BCHL c-containing Chlorobaculum (Cba.) tepidum by Donald Bryant and co-workers, most of the BCHL biosynthesis pathway in this organism was elucidated [12,13]. The C-20 methyltransferase gene, bchU, was identified in 2003 after extensive searching of the genomes of both Cba. tepidum and the green non-sulfur bacterium Chloroflexus aurantiacus. This gene was subsequently insertionally inactivated in Cba. tepidum, which resulted in chlorosomes containing BCHL d instead of BCHL c [14]. It quickly was hypothesized that if this gene was also inactivated in a BCHL e-containing organism, the mutant should produce the long-sought-after BCHL f [10]. Recently, tractable genetic systems for two BCHL e-containing strains of Cba. limnaeum were developed (one of which by our collaborator Donald Bryant), and bchU null mutants were constructed that synthesized only BCHL f [15,16]. Previous predictions concerning the spectral properties of BCHL f were confirmed. Relative to BCHL e, the absorption of aggregated BCHL f was shifted ~15 nm to shorter wavelengths, which results in an absorption maximum centered at ~705 nm at room temperature (Fig. 2.1). The absorption wavelength maximum of BCHL a in the CsmA baseplate was unaltered after bchU inactivation [15,16].
Fig. 2.1: Absorption spectra of air-saturated BChl e-containing (red line) and BChl f-containing (green line) chlorosomes at room temperature (A) and 77 K (B). The 77 K spectrum shows the CsmA-BChl α baseplate absorption is unaltered between the two types of chlorosomes [15].

An important question about chlorosomes containing BChl f concerns their energy transfer efficiency compared to chlorosomes containing BChl e. Initial characterization showed that chlorosomes containing BChl f have a lower energy transfer efficiency than the BChl e-containing parent strain [15]. A reduced efficiency of energy transfer within chlorosomes would reduce the amount of energy available to the reaction centers and might explain at least in part why BChl f has not so far been found in a naturally occurring organism. This manifests itself phenotypically as decreased growth rates at low, physiologically relevant irradiance [15]. Other
important questions concern whether the inefficiency has to do with the BChl $f$ molecule itself, its organization, or other as yet unknown reasons.

Here, we report a more complete characterization of chlorosomes containing BChl $f$, as well as photophysical parameters for monomeric BChl $f$ dissolved in pyridine and other solvents. Steady-state absorption and fluorescence spectra, circular dichroism, estimates of energy transfer efficiency, and fitting of the Gaussian simulations of the spectra to Förster’s Fluorescence Resonance Energy Transfer (FRET) theory strongly suggest that BChl $f$ is intrinsically as efficient as a light-harvesting molecule as the other BChls in the *Chlorobium* BChl family. However, because the absorption of BChl $f$ aggregates is shifted to shorter wavelengths compared to BChl $e$ aggregates, the spectral overlap between the BChl $f$ donor fluorescence and the BChl $a$ acceptor absorption in the baseplate is significantly decreased. This is proposed to be the main reason for the decreased energy transfer efficiency observed in chlorosomes containing BChl $f$.

Although the photophysical study of BChl $f$ was expanded upon in later publication [17], from the data shown here, we conclude that BChl $f$ is not only photophysically similar to BChl $e$, but is also very similar to Chl $b$, another chlorophyll containing a C-7 formyl group. From these data, we can evaluate the evolutionary consequences of BChl $f$ utilization as a major photosynthetic pigment and predict niches in which undiscovered organisms containing BChl $f$ may live.

### 2.2 Materials and Methods

#### 2.2.1 Bacterial strains, growth conditions, and chlorosome purification

Growth conditions for wild-type *Cba. limnaeum* (BChl $e$-containing), and the construction and growth of a $ΔbchU$ mutant strain (BChl $f$-containing) were previously described.
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[15]. *Cba. tepidum* was grown as described previously [18], and cells were used as a source of the BChl c that served as a reference for quantum yield measurements. Whole membranes were prepared from 3–7 day old cell cultures. Cells were centrifuged (7500 × g, 20 min) and were resuspended in 10 mM Tris–HCl buffer, pH 7.5, and were mechanically disrupted using a chilled French press operated at 138 MPa. Large cell debris and unbroken cells were removed by centrifugation (10,000 × g for 20 min). The membranes in the resulting supernatant were concentrated by ultracentrifugation at 220,000 × g for 2 h, redissolved in a minimal volume of 10 mM Tris–HCl buffer, pH 7.5, and stored until needed at 4 °C. Chlorosomes were isolated as previously described [15]. After purification, the washed and pelleted chlorosomes were resuspended in phosphate buffer (1 to 2 ml) containing 1.0 mM phenylmethylsulfonyl fluoride and 2.0 mM dithiothreitol for long-term storage at 4 °C. Before spectroscopic measurements, the chlorosomes were pelleted again by ultracentrifugation (220,000 × g for 1.5 h) and gently resuspended in 20 mM Tris–HCl buffer, pH 8.0 (1 to 2 mL).

### 2.2.2 Pigment purification

Purified chlorosomes containing BChl c or BChl f were pelleted by ultracentrifugation (220,000 × g for 1.5 h) and pigments were extracted with HPLC-grade 7:2 acetone:methanol (100 μL). The pigment solution was transferred to a microcentrifuge tube and centrifuged (13,000 × g for 5 min). The clear, green supernatant was directly injected into a Series 1100 HPLC system (Agilent Technologies Inc., Santa Clara, CA) equipped with a reverse-phase C-18 Zorbax column (4.6 × 250 mm, Agilent Technologies Inc., Santa Clara, CA) regulated at 20 °C. Pigments were eluted with 96:4 methanol:water pumped at a rate of 1 mL min⁻¹. Because there is no reason to believe that BChl c or f homologues have different optical properties (homologues differ in alkyl length at the variable C-8 and/or C-12 positions), the four main BChl
peaks (elution time between 3 and 6 min) were combined and studies were performed on a homologous mixture [19]. The pooled pigment fraction was dried under a stream of nitrogen gas and stored under positive pressure in the dark at −20 °C until needed.

2.2.3 Steady-state absorption, fluorescence, and circular dichroism measurements

Steady-state absorption and one minus transmittance (1-T, or “absorptance”) spectra for isolated chlorosomes or whole photosynthetic membranes were measured on a Lambda 950 UV/Vis/NIR spectrophotometer (Perkin Elmer Inc., Waltham, MA). All steady-state fluorescence spectra were measured using a customized PTI fluorometer (Photon Technology International Inc., Birmingham, NJ). The slits in all positions of the fluorometer were adjusted to 1 mm corresponding to a 4 nm spectral bandwidth. Fluorescence was monitored at a right angle in respect to excitation. Fluorescence excitation spectra were corrected using a calibrated reference diode. All chlorosome samples were prepared for fluorescence measurements by diluting with 20 mM Tris–HCl buffer, pH 8.0 to a Qy absorption value of 0.1. When indicated, samples were fully reduced by the addition of sodium dithionite to a total concentration of 20 mM with subsequent incubation in the dark for 1 h at 4 °C prior to measurements. Measurements at 77 K were performed after adding glycerol (final concentration, 50%, v/v) to the samples and cooling with liquid nitrogen in an Optistat DN2 cryostat (Oxford Instruments, Oxfordshire, UK).

For fluorescence emission measurements, isolated chlorosomes were excited at wavelengths corresponding to the two principal components of their absorbance bands at 457 nm and 528 nm for BChl e and at 446 nm and 508 nm for BChl f. The raw fluorescence emission spectra were scaled in order to correct for the fact that they were recorded with different excitation wavelengths (the excitation lamp has differential performance at each excitation
wavelength, and each sample has different absorption values at each excitation wavelength), allowing all spectra to be displayed on the same y-axis for a quantitative comparison. The scaling factor, \((\text{Lamp intensity } \lambda_{\text{ex}} \text{ for standard } \times \text{Ordinate } 1-T \text{ spectrum for standard at } \lambda_{\text{ex}}) / (\text{Lamp intensity } \lambda_{\text{ex}} \text{ for sample } \times \text{Ordinate } 1-T \text{ spectrum for sample at } \lambda_{\text{ex}})\), corrects for this by adjusting for the different numbers of photons absorbed by each chlorosome sample at their respective excitation wavelengths \((\lambda_{\text{ex}})\).

The 1-T spectra were recorded for a solution of BChl e chlorosomes of Abs\(721\text{ nm} = 0.1\) and for a solution of BChl f chlorosomes of Abs\(705\text{ nm} = 0.1\). The fluorescence emission spectra for chlorosomes containing BChl e from the 457 nm excitation were arbitrarily chosen to be the standard for scaling. Although this scaling is small, it is necessary to reconcile the experimental differences that arise from the use of multiple excitation wavelengths. It should also be noted that the extinction coefficient for BChl f was expected to be similar to BChl e within ~5%, just as the extinction coefficients of BChl c and d, which only differ by the C-20 methyl substituent, are quite similar (75 vs. 79 mM\(^{-1}\) cm\(^{-1}\), respectively), so no correction was made for this [9]. For fluorescence excitation spectra, dithionite-reduced whole photosynthetic membranes were used with emission monitored at 830 nm; use of whole membranes allowed for better modeling of the excitation spectrum peak of the CsmA baseplate.

Circular dichroism (CD) measurements were made on a J-815 spectropolarimeter (JASCO Inc., Easton, MD) detecting from 850-550 nm. The detector sensitivity was set to 200 mdeg, sampling resolution was set at 1 nm, sampling speed was set at 50 nm min\(^{-1}\), and integration time was set to 1 s. Four spectra were taken and signal-averaged together to reduce noise. Signal-averaged spectra were blank-corrected by manually subtracting a spectrum of pure buffer. Second derivatives of the CD spectra were calculated to facilitate peak assignment. All
samples prepared for CD were diluted to a $Q_y$ absorption of 1.0 with 20 mM Tris–HCl buffer, pH 8.0 in a 1 cm path quartz cuvette.

2.2.4 Picosecond time-resolved fluorescence measurements

Time-resolved fluorescence experiments were carried out using a Hamamatsu universal streak camera consisting of a cooled N51716-04 streak tube, C5680 blanking unit, digital CCD camera (Orca2), slow speed M5677 unit, C10647 and C1097-05 delay generators, and a 250IS imaging spectrograph from Bruker. The emitted light was dispersed on a 150 g mm\(^{-1}\) grading blazed at 800 nm. For focusing of the excitation beam on the sample and emitted light on the spectrograph, a standard optics setup A8110-01 from Hamamatsu was used. The slit at the spectrograph was set to 100 μm corresponding to 2 nm resolution.

Excitation pulses were produced by Inspire100, an ultrafast optical parametric oscillator (OPO; Radiantis-Spectra-Physics, CA, USA) pumped with Mai-Tai, an ultrafast Ti:sapphire laser, generating ~90 fs laser pulses at 820 nm with a frequency of 80 MHz. After the OPO, the pulse frequency of the excitation beam was lowered to 8 MHz by a 3980 Pulse Selector from Spectra-Physics equipped with a model 3986 controller. We assumed for BChl\(_f\) dissolved in solvents that the time interval between subsequent excitations (~125 ns) does not cause an accumulation of a pool of triplet excited state BChl\(_f\) that will overwhelm the pool of the molecules in the singlet excited state (due to competitive oxygen sensitization) over the time of the experiment, which would lead to fast disappearance of the fluorescence signal.

2.2.5 TRF data processing and global fitting

Time-resolved fluorescence data of BChl\(_f\) taken in solvents were first cleared from random noise by reconstructing the data from the dominant principal components using singular
value decomposition (SVD) [20]. Global fitting of the data sets was performed using a modified version of ASUfit, a program provided by Dr. Evaldas Katilius at Arizona State University (http://www.public.asu.edu/~laserweb/asufit/asufit.html). The full width at half-maximum of the instrument response function (IRF) was obtained as one of the global analysis parameters and was confirmed by recording a profile of the scattered excitation laser beam. Global analysis was done using an unbranched, sequential decay path model (A → B → C → D → ...) that assumes that the energy losses at each step are large enough that the reverse reaction rates are negligible (i.e., the path is irreversible). The spectral profiles obtained from this fitting of the TRF data sets are termed evolution associated fluorescence spectra (EAFS) [20]. The sequential model accurately represents the excitation decay path of individual BChl f molecules (initial internal relaxation to the lowest vibronic level of the first singlet excited state and subsequent decay of said excited state).

2.2.6 Determination of BChl f fluorescence quantum yield

For quantum yield measurements, aliquots of purified BChl c and BChl f were dried under a stream of nitrogen gas and resuspended in 1 mL of degassed (via freeze-pump-thaw) pyridine. The samples were transferred to 1 cm square quartz cuvettes with screwtops fitted with rubber septa. Before sealing the cuvettes, the headspace was sparged with nitrogen to eliminate as much oxygen as possible. Degassed (via freeze-pump-thaw) pyridine was injected through the rubber septa to adjust the absorbance of the samples at their excitation wavelengths in a series of values between 0.02 and 0.10. While this method did not result in completely anaerobic conditions, absorption spectra were taken to ensure that degradation of the pigments was negligible, as well as to accurately record the absorbance value at the excitation wavelength. Fluorescence emission spectra of BChl c and BChl f at each absorbance were taken using a 596
nm (BChl f) or 618 nm (BChl c) excitation beam, measuring emission from 625–950 nm. Fluorescence spectra were integrated to determine quantum yield using BChl c ($\Phi_F = 0.27$) as the reference using the method described in [21]. No correction for reabsorption was made because the absorbance values were low enough that the correction values are insignificant. The equation used to calculate quantum yield ($\Phi_F$) [22],

$$
\Phi_{F, \text{analyte}} = \Phi_{F, \text{standard}} \left( \frac{\text{Grad}_{\text{analyte}}}{\text{Grad}_{\text{standard}}} \right) \left( \frac{\eta_{\text{analyte}}^2}{\eta_{\text{standard}}^2} \right)
$$

Eq. 2.1

was modified to correct for the fact that the fluorescence spectra for BChl c and f were not recorded with the same excitation wavelength. The modified equation,

$$
\Phi_{F, \text{BChl f}} = \Phi_{F, \text{BChl c}} \left( \frac{\text{Grad}_{\text{BChl f}}}{\text{Grad}_{\text{BChl c}}} \right) \left( \frac{\eta_{\text{BChl f}}^2}{\eta_{\text{BChl c}}^2} \right) \left( \frac{\text{Lamp Intensity}_{\text{BChl c \text{ex}}}}{\text{Lamp Intensity}_{\text{BChl f \text{ex}}}} \right)
$$

Eq. 2.2

corrects for this by adjusting for the different numbers of photons incident on each pigment at their respective excitation wavelengths. Although this correction is small, it is necessary to reconcile the experimental differences that arise from the use of two different excitation wavelengths. Because the excitation wavelength is also the absorption maximum, and all solution sets had the same absorption value, no additional scaling factor was necessary. $\text{Grad}$ refers to the slope of a linear fit of an $x$–$y$ scatterplot of integrated fluorescence vs. absorbance and $\eta$ is the refractive index of the solvent. The $\eta$ terms cancel because both BChl c and f were dissolved in the same solvent.

### 2.2.7 Energy transfer calculations using FRET theory

The steady-state fluorescence emission spectra of the dithionite-reduced chlorosomes containing BChl e and BChl f were simulated by Gaussian functions. To apply a FRET theory
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treatment and estimate the energy transfer efficiency from main pigment to chlorosome baseplate using spectral overlap parameters, we begin with the FRET equations [9]:

\[ \phi_{ET} = \frac{1}{1 + \left( \frac{R}{R_0^6} \right)} \]  
Eq. 2.3

where \( R_0^6 = \frac{9Q_0\ln(10)\kappa^2J}{128\pi^2\eta^2N_A} \)  
Eq. 2.4

and \( J = \int_0^\infty f_D(\lambda)\epsilon_A(\lambda)\lambda^4 d\lambda \)  
Eq. 2.5

\( \phi_{ET} \) refers to the energy transfer efficiency from donor to acceptor (\( 0 \leq \phi_{ET} < 1 \)), \( R \) is the distance between donor and acceptor, \( Q_0 \) is the fluorescence quantum yield of the donor, \( \kappa^2 \) is a molecular orientation factor, \( \eta \) is the refractive index of the medium, \( N_A \) is the Avogadro’s constant, \( f_D(\lambda) \) is the fluorescence emission spectrum of the donor molecule, \( \epsilon_A(\lambda) \) is the molar extinction plot of the acceptor molecule, and \( \lambda \) is the wavelength. Making the assumption that all parameters except for the spectral overlap integral, \( J \), are equal between the two chlorosomes, Eq. 2.5 can be simplified to:

\[ \phi_{ET} = \frac{1}{1 + \left( \frac{x}{J} \right)} \]  
Eq. 2.6

in which \( x \) is a constant containing all the variables shared by the two chlorosome systems. The refractive indexes, \( \eta \), of the two chlorosome systems correspond to the hydrophobic interior of the chlorosomes, which are estimated to be essentially equal, as they contain the same basic components. We also estimated the molar extinction plot of the acceptor molecule \( \epsilon_A(\lambda) \), to be the 1-T spectrum of BChl \( a \) in the baseplate, a Gaussian with an absorption maximum at 789.3 nm and a width of 48.5 nm [23]. By calculating \( J \) for both the BChl \( e \)--BChl \( a \) pair and the BChl \( f \)--BChl \( a \) pair, we can estimate the relationship between efficiencies of the two types of chlorosomes. If, for example, we assume an efficiency level of 0.65 for BChl \( e \) to BChl \( a \)
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transfer (justified in the fluorescence excitation results of Section 2.3.2), we can calculate $x$. Using this $x$ and the specific $J$ for BChl$f$, we can calculate an estimated efficiency for BChl$f$ to BChl $a$ energy transfer.

2.3 Results

2.3.1 Steady-state absorption, fluorescence, and circular dichroism spectra

Chlorosomes containing BChl $e$ and BChl$f$ differ from those containing BChl $c$ and $d$ because the Soret bands of the BChl oligomers are noticeably split into two components in the absorption spectra at room temperature (see Fig. 2.1 and Chapter 1, Fig. 1.8). The nature of this splitting has been previously explored computationally [24]. It was found that two orthogonally polarized Soret transitions, $B_x$ and $B_y$, were mostly responsible for the two Soret components. To explore experimentally the fluorescence contribution of each of those particular Soret bands in our chlorosome samples experimentally, each band was individually excited and fluorescence emission spectra were obtained. Because these are Soret bands of the BChl oligomers, we expected that their fluorescence emission would resemble that of BChl oligomers excited into a Q band, or through energy transfer, the BChl $a$ baseplate. Figs. 2.2 and 2.3 correspond to the fluorescence emission spectra obtained from exciting the two Soret band components of chlorosomes containing BChl $e$. The fluorescence emission spectra at both room temperature and 77 K are nearly identical and represent fluorescence emission from both oligomers and the baseplate; the emissions match well with the earlier calculations [24]. It should be noted that the small amount of fluorescence emission at 675 nm observed in Fig. 2.3A is probably due to a small amount of monomeric BChl $e$ present (in the chlorosomes) that absorbs at the excitation wavelength for the experiment. The low-temperature fluorescence emission spectra (Figs. 2.2B and 2.3B) show a shift to longer wavelengths for all emission maxima. This is due in part to a
perturbation (which confers stability) caused by the change of environment polarizability (liquid to glass) at low temperatures. Because the $Q_y$ transition is a collection of excitonic transitions (as evidenced later in Fig. 2.7), this shift is also attributed to a redistribution of the excitonic population probability to lower energies at lower temperatures. The scaling factors introduced Section 2.2.3 changed the spectra minimally; the factor varied between 0.90 and 1.01.

**Fig. 2.2:** Fluorescence emission spectra for air-saturated (dashed lines) and dithionite-reduced (solid lines) BChl $e$-containing chlorosomes at room temperature (A) and 77 K (B). The excitation wavelength was into the shorter-wavelength Soret component at 457 nm for each.
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Fig. 2.3: Fluorescence emission spectra for air-saturated (dashed lines) and dithionite-reduced (solid lines) BChl e-containing chlorosomes at room temperature (A) and 77 K (B). The excitation wavelength was into the longer-wavelength Soret component at 528 nm for each.

Figs. 2.4 and 2.5 show the corresponding fluorescence emission spectra that result from excitation of the two Soret band components of chlorosomes containing BChl f. A different trend is observed here. The fluorescence emission resulting from excitation of the shorter wavelength Soret component (Fig. 2.4, centered at 446 nm) in air-oxidized conditions contains significantly higher amplitude of fluorescence from BChl f monomers. It should also be noted that any BChl f monomers that are present will also absorb at this excitation wavelength, but according to the absorption spectrum shown in Fig. 2.1, this should be minimal. The emission contribution from
the BChl*f monomers is much higher than expected, indicating that there is a different energy-level mixing in these chlorosomes than would be expected from the calculations in [24]. In contrast, the fluorescence emission from the longer wavelength Soret component (Fig. 2.5 centered at 508 nm) corresponds nearly identically in pattern to the counterpart for BChl e. It should also be noted that for excitation in the shorter wavelength Soret band at low temperatures (Fig. 2.4B), the fluorescence emission from the BChl*f oligomers is split into two distinct components centered at 704 nm and 747 nm [15]. The component emitting at 704 nm corresponds to a low-energy vibronic level of the BChl*f monomer, and the component emitting at 747 nm corresponds to the fluorescence emission from BChl*f oligomers. All preparations of chlorosomes containing BChl*f had lower fluorescence emission amplitudes from the BChl a-, CsmA-containing baseplate than chlorosomes containing BChl e having the same absorbance in the Qy band.
**Fig. 2.4:** Fluorescence emission spectra for air-saturated (dashed lines) and dithionite-reduced (solid lines) BChl $f$-containing chlorosomes at room temperature (A) and 77 K (B). The excitation wavelength was into the shorter-wavelength Soret component at 446 nm for each.
Fig. 2.5: Fluorescence emission spectra for air-saturated (dashed lines) and dithionite-reduced (solid lines) BChl$_f$-containing chlorosomes at room temperature (A) and 77 K (B). The excitation wavelength was into the longer-wavelength Soret component at 508 nm for each.

Fig. 2.6 shows the CD spectra of chlorosomes containing BChl $e$ and BChl $f$, along with their second derivatives. The spectra in Fig. 2.6A appear to be consistent with those for molecular species exhibiting exciton-coupled energy transfer processes, indicating that the main light-harvesting pigments in each type of chlorosome are strongly coupled. The second derivatives in Fig. 2.6B confirm that the CD spectra in Fig. 2.6A are centered upon their respective absorption maxima. The shapes of the second derivatives further suggest the presence of only a single spectral component in each type of chlorosome.
Fig. 2.6: Circular dichroism spectra (A) and their second derivatives (B) of BChl e-containing (red line) and BChl f-containing (green lines) chlorosomes in dithionite-reduced buffer.

2.3.2 Fluorescence excitation spectra and efficiency estimates

Fig. 2.7 shows the fluorescence excitation spectra of intact photosynthetic membranes of WT *Cba. limnaeum* (containing BChl e, Fig. 2.7A) and of the Δ*bchU* mutant (containing BChl f; Fig. 2.7B) overlaid with the corresponding 1-Τ spectra. By normalizing the amplitude of the two spectra at the excitation maximum of the lowest-energy acceptor molecule, the energy transfer efficiency can be estimated. It is assumed here that energy transfer efficiency from the chlorosome baseplate to the acceptors in the membrane (i.e., the Fenna-Matthews-Olson
complex and the type I reaction center) is the same between the two types of chlorosomes, and that it is close to 100%. Under this assumption and the conditions employed, Fig. 2.7A shows that BChl *e* oligomers are able to transfer energy with an overall efficiency of ~65%. In contrast, BChl *f* oligomers are only able to transfer energy with an efficiency of ~39% (Fig. 2.7B).

![Figure 2.7](image)

**Fig. 2.7**: Fluorescence excitation spectra (dashed lines) overlaid with the respective 1-*T* spectra (solid lines) of BChl *e*-containing (A) and BChl *f*-containing (B) chlorosomes. The emission wavelength was 820 nm.
2.3.3 Energy transfer calculations using FRET theory

Table 1 shows the Gaussian simulation parameters for the fluorescence emission of dithionite-reduced BChl e and f chlorosomes from this study, compared with those from BChl c and d chlorosomes from another study [23]. The data from Table 2.1 indicate that the peak widths of fluorescence components are very similar between chlorosomes of different pigment compositions. Even when the main light-harvesting pigment changed from BChl c to BChl d, BChl e, or BChl f, the fluorescence emission from the BChl a-containing CsmA baseplate had essentially the same maximal wavelength. As mentioned in Section 2.2.7, absorption of the BChl a-containing baseplate component can be estimated as a Gaussian centered at 789.1 nm with a width of 48.5 nm [23]. Thus, a direct comparison of the overlap between BChl e and the baseplate and BChl f and the baseplate can be made. This comparison is depicted in Fig. 2.8.

Table 2.1: Fitting parameters for Gaussian simulations of fluorescence emission spectra of fully reduced, isolated chlorosomes containing BChl c, d, e, or f.

<table>
<thead>
<tr>
<th>Main Pigment Gaussian:</th>
<th>BChl c</th>
<th>BChl d</th>
<th>BChl e</th>
<th>BChl f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude</td>
<td>0.972</td>
<td>0.998</td>
<td>0.960</td>
<td>0.951</td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td>775.7</td>
<td>759.8</td>
<td>744.1</td>
<td>730.2</td>
</tr>
<tr>
<td>Width (nm)</td>
<td>44.1</td>
<td>43.8</td>
<td>36.6</td>
<td>36.1</td>
</tr>
<tr>
<td>Baseplate Gaussian:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplitude</td>
<td>0.304</td>
<td>1.363</td>
<td>0.597</td>
<td>1.065</td>
</tr>
<tr>
<td>Wavelength (nm)</td>
<td>810.1</td>
<td>807.3</td>
<td>812.5</td>
<td>813.3</td>
</tr>
<tr>
<td>Width (nm)</td>
<td>29.3</td>
<td>30.1</td>
<td>44.4</td>
<td>40.4</td>
</tr>
</tbody>
</table>

Each spectrum was normalized to a maximum of 1.0 for the BChl c, d, e, or f emission peak.

\(^a\) From Gausgrove et al., 1992.

\(^b\) From this work.
Fig. 2.8: Gaussian simulations and spectral overlap comparison of BChl $e$-containing (A) and BChl $f$-containing (B) chlorosomes.

This analysis shows that the calculated mathematical overlap area for BChl $f$ oligomers and the baseplate is 47% smaller than that for BChl $e$ oligomers and the baseplate. The basic mathematical overlap area, however, cannot be used in a FRET theory treatment of energy transfer from major BChl to the baseplate in the system. Rather, the $J$ function described in Eq. 2.5 must be used. Table 2.2 summarizes the relevant overlap factors determined in this study. Noting from Fig. 2.7A that the efficiency of BChl $e$-BChl $a$ transfer in BChl $e$ chlorosomes is $\sim 65\%$, the FRET efficiency of BChl $f$-BChl $a$ transfer in BChl $f$ can be calculated to be $41\%$. 
This calculation is in excellent agreement with the experimental determination (39%) as described in Section 2.3.2 and as shown in Fig. 2.7B.

Table 2.2: Spectral overlap factors for energy transfer from chlorosomes containing BChl e or f.

<table>
<thead>
<tr>
<th></th>
<th>BChl e</th>
<th>BChl f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overlap area</td>
<td>1.00</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>between main pigment and BChl a</td>
<td>relative to BChl e from basic spectral integration</td>
</tr>
<tr>
<td>FRET theory J factor</td>
<td>$3.32 \times 10^{12}$</td>
<td>$1.25 \times 10^{12}$</td>
</tr>
<tr>
<td>Estimated FRET</td>
<td>0.65</td>
<td>0.41</td>
</tr>
<tr>
<td>efficiency from</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pigment to BChl a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>using Eq. (6)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Overlap factors were determined from the emission parameters of Table 2.1 and a Gaussian for the BChl a absorption in the baseplate centered at 789.1 nm with a width of 48.5 nm. The baseplate Gaussian parameters are taken from Causgrove et. al., 1992. The efficiency value of 0.65 for BChl e is an estimate taken from Fig. 2.7

2.3.4 Singlet excited state dynamics of monomeric BChl f

Fig. 2.9 shows the results of a determination of quantum yield for BChl f that was determined by comparison with a previously characterized BChl c standard [21]. The fluorescence spectra of the concentration series of BChl c and BChl f are shown in Fig. 2.9A. By integrating each spectrum and converting these data to a plot of integrated fluorescence vs. absorbance at the excitation wavelength for each member of the series, an x–y scatter plot can be constructed. A linear regression of each data set yields a slope (gradient) that can be used in Eq. 2.1. Upon inserting the appropriate values into Eq. 2.2, the fluorescence quantum yield of BChl f was found to be 0.13, a value about 50% less than that of BChl c.
Fig. 2.9: The corrected fluorescence emission for a concentration series of BChl \( c \) (black lines) and BChl \( f \) (green lines) (A), along with the linear correlation of integrate fluorescence vs. absorbance for determination of the fluorescence quantum yield of monomeric BChl \( f \) (B). BChl \( c \) was used as the known quantum yield reference. The solvent for all measurements was degassed pyridine.

Steady-state absorption, steady-state fluorescence, and the time-resolved fluorescence of monomeric BChl \( f \) in organic solvents are shown in Fig. 2.10. Three different solvents were used: Pyridine (Pyr, hexa-coordinates the central Mg), diethyl ether (DE, penta-coordinates the central Mg), and 2-methyltetrahydrofuran (2-MeTHF, hexa-coordinates the central Mg, can be frozen into a glass at 77 K). After globally-fitting the data, a single spectral-kinetic EAFS component is found for each, with a lineshape identical to the steady-state fluorescence spectrum. The lifetime of this single component represents the singlet excited-state lifetime for
BChl $f$ in each solvent. These lifetimes are comparable to the previously determined singlet excited-state lifetime of 2.9 ns for BChl $e$ in pyridine and significantly less than the lifetimes observed for BChl $c$ and $d$ (6.7 and 6.3 ns, respectively) in pyridine [31].

**Fig. 2.10:** Spectroscopic properties of BChl $f$ in pyridine at room temperature (A-C), diethyl ether at room temperature (D-F), and 2-methyltetrahydrofuran at 77 K (G-I). Steady-state absorption and fluorescence spectra are shown in the top panels (A, D, G), TRF streak camera images are shown in the middle panels (B, E, H), and the global fitting spectral-kinetic components with lifetime are shown in the bottom panels (C, F, I).

The difference in central-Mg coordination between penta-coordinate (DE) and hexa-coordinate (Pyr and 2-MeTHF) results in differences in resolution of certain spectral components (see Fig. 2.10A, D, and G). In hexa-coordinate environments, the Q$_x$ and Soret absorption bands show enhanced resolution. When moving to cryogenic temperatures (in the case of 2-MeTHF) in
hexa-coordinate environments, the Soret band shows additional distortion, possibly indicating partial resolution of the $B_x$ and $B_y$ bands in the 460-480 nm range.

At cryogenic temperatures, the singlet excited state lifetime of $\text{BChl}_f$ increases from 3.4 ns to 5.1 ns, demonstrating that lowering the temperature reduces the coupling of the ground and excited states. The main fluorescence band at cryogenic temperatures is also shifted to lower energies by about 2 nm.

### 2.4 Discussion

This study sought to further understand the similarities and differences between chlorosomes containing the naturally occurring pigment, $\text{BChl}_e$, and chlorosomes from a $\Delta bchU$ mutant of *Cba. limnaeum* that contain $\text{BChl}_f$. A previous study established that the *Cba. limnaeum* mutant producing $\text{BChl}_f$ instead of $\text{BChl}_e$ is viable, although its doubling time is about 40% slower under low irradiance conditions [15]. Sufficient data now exist to explain this phenotypic difference from a biophysical perspective.

Although variations in the relative contribution of fluorescence from oligomer $\text{BChl}_{e/f}$ vs. baseplate $\text{BChl}_a$ based on excitation wavelength can be seen, fluorescence emission spectra show that chlorosomes containing $\text{BChl}_f$ consistently display a lower fluorescence emission from the CsmA baseplate than chlorosomes containing $\text{BChl}_e$. These variations may indicate that slightly different oligomeric structural types of $\text{BChl}$ exist between these chlorosomes. Perhaps a better indication of structural difference is the narrower absorption bandwidth of the $\text{BChl}_f$ aggregates in the mutant chlorosomes vs. the $\text{BChl}_e$ aggregates in the WT. This lessened inhomogeneous broadening likely means that the BChl oligomerization regime is more regular throughout the entirety of the chlorosome in the $\Delta bchU$ mutant than in the WT.
Additionally, after air oxidation, chlorosomes containing BChl $f$ also consistently showed a higher fluorescence emission from BChl $f$ monomers. This indicates one way in which energy transfer in chlorosomes containing BChl $f$ is inhibited in comparison to the BChl $e$ system. This result is strongly supported by the fluorescence excitation experiments, which are a more direct measurement of energy transfer events. As mentioned in Section 2.3.2, Fig. 2.7 shows that the chlorosomes containing BChl $f$ transfer energy nearly 40% less efficiently to the remainder of the photosynthetic apparatus than chlorosomes containing BChl $e$.

With only these results in hand, it is reasonable to ask whether this efficiency decrease is due to less optimal absorption properties of the BChl $f$ molecule, substantial differences in its oligomerization and organization, a decreased spectral overlap with the BChl $a$ acceptor in the baseplate, or a combination of all three. The BChl $e$ and BChl $f$ molecules only differ structurally by a single methyl group at the C-20 position, but it has long been known that even minor differences in the substituents of chlorin or bacteriochlorin rings can appreciably change their spectroscopic and structural properties, possibly by puckering of the chlorin ring [25,26]. Examples include the Chl $a$ and Chl $b$ pairs, as well as the BChl $a$ and BChl $b$ pairs [9]. The quantum yield and lifetime measurements, circular dichroism spectroscopy, and FRET theory calculations performed here serve to address this question.

Despite the fact that BChl $c$, $d$, $e$, and $f$ belong to the same “Chlorobium chlorophylls” family, they do not share all spectroscopic properties in organic solvent. Looking at excited state lifetimes and fluorescence quantum yields measured here and elsewhere, it is clear that these pigments can be divided into two sub-groups. Those pigments with a methyl group at the C-7 position (BChl $c$ and $d$) share a singlet excited state lifetime and fluorescence quantum yield twice that of those with a formyl group at the C-7 position (BChl $e$ and $f$). We can even expand
the group to all natural chlorin-type pigments (adding Chl \(a, b, d, \) and \(f\) to the group of BChl \(c, d, e, \) and \(f\)), preserving this trend [27,28].

Considering that the majority of the BChls in functional chlorosomes are organized into oligomers that are strongly exciton-coupled (as evidenced by the absorption spectra in Fig. 2.1 and the CD spectra in Fig. 2.6), the relatively small differences in the fluorescence quantum yield or lifetimes of monomers should not, by themselves, be responsible for the major differences in energy transfer observed for the BChl oligomers in the chlorosomes and cells of the \(\Delta bchU\) mutant.

The circular dichroism results show similarity in the excitonic coupling of the pigment oligomeric structures in chlorosomes containing BChl \(e\) and BChl \(f\). Both types of chlorosomes show strong excitonic coupling (as evidenced by an S-shaped curve in the \(Q_y\) region), which leads to long-range energy transfer across the oligomeric structures and ultimately facilitates energy transfer to the BChl \(a\) associated with the CsmA in the baseplate. While the time-dependent rates of this energy transfer are explored in later studies [17], it can be concluded from the findings in this study that chlorosomes containing BChl \(f\) are functionally quite similar to their counterparts containing BChl \(c, d, \) or \(e\). Therefore, we conclude that monomeric BChl \(f\) is similar to these other three BChls and that the differences observed for chlorosomes containing BChl \(f\) are not the result of extensive differences in oligomeric excitonic coupling of the BChls or to fundamental differences in the biophysical properties of BChl \(f\).

The FRET analysis of the two systems, however, identified a major difference and the possible source of the energy transfer inefficiency. The observed 47% decrease in overlap for the donor and acceptor pair when replacing BChl \(e\) with \(f\) corresponds to a 40% decrease in overall FRET efficiency, assuming that the relative concentrations of donor and acceptor are the same.
between the two chlorosome systems. Because our estimates of differences in energy transfer efficiency from the FRET analysis match quite well with the estimate from fluorescence excitation, we conclude that the observed decreased efficiency in energy transfer in chlorosomes containing BChl $f$ is primarily due to the decreased spectral overlap between BChl $f$ oligomeric donors and BChl $a$ acceptors. Considering the otherwise high degree of similarity observed for chlorosomes containing BChl $e$ or BChl $f$, this large decrease in the spectral overlap is currently the only identified parameter that can explain the very large decrease in energy transfer efficiency reported here. This is manifested as a two-fold slower growth rate at low light intensity [15], which would not allow organisms producing BChl $f$ to compete favorably in the same niches with organisms synthesizing BChl $e$.

### 2.5 Concluding remarks

This chapter reports the results from a detailed spectroscopic analysis of chlorosomes containing BChl $f$ from a Δ$bchU$ mutant of the GSB Cba. limnaeum. The slow growth rate of this mutant at low irradiance can be attributed in part to decreased efficiency of energy transfer within the chlorosomes from BChl oligomer to baseplate. This decreased efficiency, in turn, can be mostly attributed to a decreased spectral overlap between the oligomeric BChl $f$ donors and BChl $a$ acceptors associated with the chlorosome baseplate, as compared to the oligomeric BChl $e$ donors and the same BChl $a$ acceptor in WT chlorosomes. Our manuscript published shortly after the results of this chapter (Niedzwiedzki et. al., 2014, [17]) describes additional ultrafast optical spectroscopy which corroborates the data here. These results further illustrate the photophysical similarities between BChl $f$, BChl $e$, and Chl $b$ (which all share a formyl group at the C-7 position). Further studies, such as high-resolution microscopy, will complement these findings and may possibly elucidate additional causes for the decreased efficiency of energy transfer.
transfer (i.e., slight variations in BChl oligomer structures). Further experiments must also be done to explain the unusually high fluorescence amplitude from monomeric BChlf that occurs within chlorosomes containing BChlf after exposure to oxygen. There may be an unresolved structural rearrangement or mixing of absorption bands occurring in the Soret region that could explain this observation.

Although they have thus far eluded discovery, there is no fundamental reason why organisms containing BChlf could not be found in nature. The ΔbchU mutation is not lethal, and the associated growth defects for cells producing BChlf are similar in magnitude to those observed for organisms that synthesize BChld instead of BChlc [14]. However, any green sulfur bacterium producing BChlf would probably require a high irradiance niche; otherwise it would have to compete with organisms producing BChle that would probably coexist in the same environments. Such an organism would also probably receive light that would be filtered by organisms containing Chl a, Chl b, and possibly Chld. Lastly, the niche would need to be anoxic and contain either thiosulfate or sulfide, or both. High irradiance environments devoid of these filtering effects and atmospheric oxygen are probably rare, and the potential competitors are numerous and diverse. An example of such a niche could be a sun-exposed, anoxic, saline lagoon devoid of plant or algal life, such as those found in land-locked desert salt lakes. A certainty is that organisms potentially producing BChlf have had a very long time, perhaps >2 billion years, to adapt to such niches. So, it remains possible that organisms producing BChlf may be found in nature.

2.6 References

Chapter 2


Chapter 2


Chapter 3: Self-Assembly and Energy Transfer in Biohybrid Chlorosome-like Light-Harvesting Complexes

3.1 Introduction

As described in Chapter 1, knowledge of the biogenesis pathway of the chlorosome is scarce. While different scenarios have been proposed, a common theme is clear: the injection of thousands of BChl c, d, or e molecules (also known as the “Chlorobium chlorophylls”) into the growing chlorosome is driven by hydrophobic forces that attract and keep these BChls self-assembled inside. Capturing and replicating this self-assembly process \textit{in vitro} has been attempted for over 30 years \cite{1}. Various methodologies have been developed using solid films, binary solvent mixtures, addition of natural lipid, addition of detergents, addition of carotenoids, and \textit{de novo} synthesis of pigment analogs \cite{2–5}. These attempts met with mixed results, and the likely self-assembled molecular coordination structure in natural chlorosomes has only been demonstrated in the past five years \cite{6–8}.

A methodology has recently been developed \cite{9} to create light-harvesting dyes using an amphipathic diblock copolymer to create micelle-like structures in which hydrophobic pigment molecules are forced into the inside of the micelle, which itself is suspended in aqueous medium. The diblock copolymer is a mixture of two polymers built from repeating units of butadiene and ethylene oxide where the linkage is either 1’-4’ or 1’-2’. The structure of the diblock copolymer is shown in Fig. 3.1. We have amended the aforementioned methodology for use in constructing
biohybrid chlorosome-like structures. We hypothesize that the diblock copolymer mimics the natural lipid monolayer envelope found as the exterior of natural chlorosomes.

Most chlorosomes from natural sources only contain one type of self-assembling “Chlorobium chlorophyll” [1,10,11] Few examples where two types of these molecules occupy the same chlorosome are known. In one such example, a frameshift mutation within the \textit{bchU} gene, whose product methylates the C-20 position on the BChl ring, intermittently switches the final pigment product from BChl \textit{c} to \textit{d} [12–14]. A tantalizing question can be posed: why does nature generally choose to incorporate only one type of “Chlorobium chlorophyll?” In principle, having all the “Chlorobium chlorophylls” within a single chlorosome would extend the viable spectral range for light harvesting and therefore extend the niches the organism could be found in.

There were two goals for this study: (1) to investigate whether we can create tunable biohybrid chlorosome-like light harvesting complexes using a combination of natural pigments and synthetic diblock copolymer, and (2) to investigate whether multiple types of “Chlorobium chlorophylls” (including BChl \textit{f} from the \textit{Chlorobaculum limnaeum ΔbchU} mutant described in Chapters 1 and 2) can be inserted into the same complex, while maintaining self-assembly and energy transfer. Addition of BChl \textit{a}, the pigment found universally in the chlorosome’s baseplate complex, into the biohybrid chlorosomes (even though it cannot self-assemble like BChls \textit{c}, \textit{d}, \textit{e}, and \textit{f}) was also investigated. This study represents the advancement of a platform for building light-harvesting dyes that have tunable absorption spectra, energy transfer character, size, and shape. Although it has not been tested, functionalization of the hydrophilic end of these diblock copolymers theoretically would provide additional framework in which these dyes can be attached to surfaces.
3.2 Materials and Methods

3.2.1 Bacterial strains, growth conditions, and pigment purification

Each bacteriochlorophyll used in this study was extracted and purified from a different organism. *Chlorobaculum tepdium* TLS (for BChl c) and *Chlorobaculum tepidum ΔbchU* (for BChl d, a gift from Dr. Donald Bryant, The Pennsylvania State University) were anaerobically grown in 250 mL Wheaton bottles using GSB media described previously at 40 °C in 100 μE light for 4 days [12,15]. *Pelodictyon phaeum* (for BChl e) was grown identically to *Chlorobaculum tepidum* TLS, except for the addition of extra sodium chloride in the medium to 4% total (w/v) and a decrease in growth temperature to 30 °C [16]. *Chlorobaculum limnaeum ΔbchU* (for BChl f, a gift from Dr. Donald Bryant, The Pennsylvania State University) was grown in 250 mL Wheaton bottles using GSB media described previously at 40 °C in 100 μE light for 7 days [17]. *Rhodobacter capsulatus* SB1003 (for BChl a, a gift from Dr. Robert Kranz at WUSTL) was grown semi-aerobically (1 L media in a 2.8 L Fernbach flask) using RC-V media at 32 °C in the dark for 2 days shaking at 60 rpm [18].

All cultures were pelleted via centrifugation at 11,600 ×g and stored as wet pellets at -20 °C until needed. To extract the pigments, frozen cell pellets were re-suspended using minimal 7:2 HPLC-grade acetone:methanol and vortexed thoroughly. The solution was centrifuged for 5 minutes at 13,000 ×g, the supernatant decanted and filtered through a 0.22 μm syringe filter. The filtered solution was directly injected in 100 μL aliquots into an 1100 Series HPLC (Agilent Technologies Inc., Santa Clara, CA) equipped with a fraction collector and reverse-phase C-18 Zorbax column (4.6 × 250 mm, Agilent Technologies Inc., Santa Clara, CA) regulated at 20 °C. Pigments were eluted with 60:36:4 acetonitrile:methanol:tetrahydrofuran pumped at a rate of 1.5 mL min⁻¹. The four main peaks for BChl c (4.0 – 6.25 min), BChl d (3.75 – 6.0 min), BChl e
(3.25 – 5.25 min), and BChl\textsubscript{f} (3.0 – 5.25 min) corresponding to spectrally identical homologs differing in alkylation at the C-8 and C-12 positions were collected. Only one BChl\textsubscript{a} peak (10.5 – 12.0 min) was collected. For each pigment, multiple runs were pooled until 1 mg of total for each pigment (calculated via Beer’s Law) was collected. Pure pigment was dried under a stream of Ar gas, transferred to an anaerobic chamber and sealed under positive pressure, then stored at -20 °C until needed.

Chlorosomes from GSB isolate HL-130-GSB that can produce and use both BChl\textsubscript{c} and \textit{d} for light-harvesting was also acquired as a gift from Dr. Donald Bryant and co-workers at The Pennsylvania State University. This strain was isolated in 2012 from a cyanobacteria-dominated mat in Hot Lake, a magnesium sulfate-rich meromictic salt lake in central Washington State, USA [19]. Characteristics and growth conditions for the strain will be reported elsewhere. The chlorosomes were harvested as reported previously [17] and shipped to Washington University in St. Louis for analysis. The chlorosomes were reduced by addition of sodium dithionite to 10 mM final concentration and tightly sealed from air prior to any spectroscopic characterization.

3.2.2 Self-assembly of natural bacteriochlorophylls with diblock copolymer

Hydroxyl-terminated poly(ethylene oxide)-block-poly(butadiene) (PEO-b-PBD) with block weights of 1.3 and 1.2 kDa and a polydispersity index of 1.1 (product # P6723-BdEO) was purchased from Polymer Source Inc. (Quebec, Canada). The semisynthetic chlorosome constructs were prepared at Los Alamos National Laboratory by slowly infusing polymer and pigments dissolved in tetrahydrofuran (THF) with Tris-HCl buffer. Briefly, PEO-b-PBD, BChl\textsubscript{c}, \textit{d, e, f}, or \textit{a} (or combinations thereof) were mixed from stock solution in THF to give final ratios of 15:1 pigment:polymer. 20 mM Tris buffer, pH 8, was added at 2 mL hr\textsuperscript{-1} to samples with stirring. The final concentration of THF did not exceed 5% of the total solution volume. Samples
were then purified on continuous sucrose (10-30%) gradients containing 20mM Tris-HCl buffer, pH 8.0, at 250,000 x g for 15 hours and 4°C. Sucrose was removed via buffer exchange using Amicon 50 MWCO centrifugal filters (EMD Millipore, Billerica, MA).

### 3.2.3 Steady-state optical spectroscopy and spectropolarimetry measurements

All samples were adjusted with 20 mM Tris-HCl buffer, pH 8.0, until they had a volume of 500 μL and a Qy band absorbance value of 0.2 in 1 cm × 0.5 cm plastic cuvettes. Room temperature steady-state absorbance measurements were performed using a UV-1800 spectrophotometer (Shimadzu Corp. USA, Columbia, MD). Cryogenic steady-state absorbance measurements were performed using a liquid nitrogen Optistat DN-2 optical cryostat (Oxford Instruments, Oxfordshire, UK) and a Lambda 950 Series UV-Vis-NIR spectrophotometer (Perkin-Elmer Inc., Waltham, MA). Before cryogenic experiments, 500 μL of pure glycerol was added to each sample, resulting in a total volume of 1 mL, Qy absorbance value of 0.1 and glycerol concentration of 50% (v/v).

Steady-state fluorescence emission was performed using the customized PTI fluorometer (Photon Technology International Inc., Birmingham, NJ) described in Chapter 2. Excitation was performed at 515 nm (to selectively excite BChl e or f, if they were present) or at 450 nm (to selectively excite BChl c or d, if e and f were not present), ensuring that the excitation priority was given in the following order: BChl f; e; d; c.

Circular dichroism spectra were collected using a J-815 spectropolarimeter (JASCO Inc., Easton, MD), scanning from 850 nm to 350 nm. The detector sensitivity was set to 200 mdeg, sampling speed was set to 50 nm min⁻¹, bandwidth set to 2 nm, and integration time set to 1 s. Four scans of each sample were signal-averaged to obtain noise correction and the averaged spectra were then manually blank-corrected using the spectrum of pure buffer.
3.2.4 Picosecond time-resolved fluorescence measurements

Time-resolved fluorescence measurements were carried out using the setup described in Chapter 2 with minor modification. Excitation pulses were generated at 512 nm with a final frequency of 8 MHz (125 ns between subsequent excitations) and intensity of 0.17 mW, corresponding to a photon flux of $1 \times 10^{10}$ photons cm$^{-2}$ per pulse. In the streak camera, both the fast speed M5676 single sweep unit and the slow speed M5677 single sweep unit were used and interchanged when different temporal resolution was necessary.

3.2.5 Transient data processing and global fitting

The time-resolved fluorescence spectra were cleared from random noise by recomposing the data from the dominant principal components using the singular value decomposition (SVD) function in SurfaceXplorer (Ultrafast Systems LLC, Sarasota, FL). Attempts were made to globally fit the spectra with a combination of monoexponentially decaying spectral-kinetic components convoluted by Gaussian approximation of the instrument response function (IRF). The IRF was collected by measuring the profile of light scattered onto the detector with no sample in the system. Global fitting was satisfactorily accomplished in the chlorosome sample that naturally contains a mixture of BChl $c$ and $d$, but, this could not be done in the biohybrid constructs; each spectral set contained second-order decay components that could not be fit. Therefore, only representative spectral or kinetic traces of those data are shown.

3.2.6 Sizing and structural characterization

Transmission electron microscopy was performed at Los Alamos National Lab on a low energy LVEM5 electron microscope (Delong America, Montreal, Canada). Samples were drop-
cast on onto copper grids (Cu-300, Pacific Grid-Tech, San Francisco, CA) and dried in a vacuum desiccator overnight before analysis.

## 3.3 Results

### 3.3.1 Appraisal of self-assembly and self-assembly combinations

Sixteen constructs were created using the self-assembly methods mentioned in section 3.2.2. These represent all possible unary, binary, ternary, and quaternary combinations of BChl c, d, e, and f in equimolar amounts, plus one variable stoichiometry example, and one quinary combination that incorporated BChls c, d, e, f mixed with a sub-stoichiometric amount of BChl a. The experimental scheme for self-assembly is shown in Fig. 3.1A. In this model, the constructs mimic natural chlorosomes in form, except that the natural lipid monolayer is replaced with self-assembled diblock copolymer. The chlorosome baseplate is also absent because no protein has been added to the constructs. Fig. 3.1B shows sucrose gradients of the ternary, quaternary, and quinary self-assembled constructs. It is interesting to note that all constructs containing BChl e show two populations of constructs that differ in density (or more generally, size). In the construct lacking BChl e, only one, heavy population is seen. In the constructs which contain two populations, the absorption spectra of the two populations are identical (data not shown). Therefore, for consistency, we studied only the heavy populations of each construct. The heavy populations were sized with electron microscopy (Fig. 3.1C), and were shown to be ellipsoids measuring between 150-200 nm in length.
Figure 3.1: (A) An experimental scheme for construct self-assembly using natural pigment and diblock copolymer. (B) Sucrose density gradients (20-40% sucrose) of the ternary, quaternary, and quinary constructs. (C) Example electron microscopy image of an individual self-assembled construct (measurement bar = 500 nm scale).

3.3.2 Steady-state spectroscopy and spectropolarimetry measurements

The absorbance spectra of each of the sixteen constructs, including fluorescence emission spectra, are included in Figs. 3.1, 3.2, and 3.3. In each case of fluorescence, excitation is preferentially given to BChl $e$ or $f$ (the most blue-shifted pigments in the Q$_y$ region and most red-shifted pigments in the Soret region) at 515 nm. If BChl $e$ or $f$ is not present in the complex, then BChl $d$ or $c$ is excited at 450 nm. In each of Figs. 3.2, 3.3, and 3.4, the spectra of the unary mixtures (most resembling natural chlorosomes) are shown to facilitate comparison.
Figure 3.2: Normalized absorption spectra (left panel) and fluorescence emission spectra (right panel) of unary and binary pigment combinations in the biohybrid chlorosome constructs. Unary constructs are offset by 1 a.u. to facilitate comparison.
Figure 3.3: Normalized absorption spectra (left panel) and fluorescence emission spectra (right panel) of unary and ternary pigment combinations in the biohybrid chlorosome constructs. Unary constructs are offset by 1 a.u.

Figure 3.4: Normalized absorption spectra (left panel) and fluorescence emission spectra (right panel) of unary, quaternary, and quinary pigment combinations in the constructs. Unary constructs are offset by 1 a.u.
Each unary mixture construct well approximates the absorption spectrum of a natural chlorosome of each type. Interestingly, the BChl e unary construct’s absorption maximum in the Q_y region matches quite well with that from the BChl f unary construct. In wild-type *Chlorobaculum limnaeum*, the BChl e-containing chlorosome absorbs maximally in the Q_y region at about 720 nm, about 15 nm to lower energy than that in our biohybrid construct. But, the BChl e used comes from *Pelodictyon phaeum*, which has a chlorosome that absorbs maximally closer to 700 nm than *Chlorobaculum limnaeum*. The only difference between these two organisms’ BChl e is the distribution of homologs differing in alkylation at the C-8 and C-12 positions; it would seem that the distribution of this alkylation extent can tune the absorption maximum to a similar level as the methylation at C-20 or the formylation at C-7.

Fig. 3.5 shows the effect of using a stoichiometric combination of pigments other than a 1:1 ratio. The absorption spectrum of a construct can be finely tuned by simply altering pigment ratios, although a 1:1 ratio results in a wider absorption band.
Fig. 3.5: Normalized absorption spectra of two stoichiometric combinations of BChl c and BChl e pigments in the same construct. The inset shows the Q_y spectral region in detail, labeled with the wavelengths of maximal absorption.

In each construct, fluorescence is seen exclusively from the pigment absorbing at the lowest Q_y energy, indicating that energy will flow in the general direction from BChl_f → e → d → c. From these experiments, it is impossible to ascertain the level of singlet-singlet annihilation, or aggregate-induced quenching, in the system; we can only conclude that at least some of the energy absorbed in the higher-energy pigments is eventually transferred to the lowest without being emitted via fluorescence along the way. To our surprise, in the quinary mixture construct containing BChl a in small stoichiometric amounts, a sizeable fluorescence
shoulder is seen around 800 nm, indicating that energy can transfer from the self-assembling BChls to the presumably non-self-assembling BChl a.

Fig. 3.6 shows circular dichroism spectra for each construct. These spectra vary widely, yet each generally contains the sigmoidal-shaped responses that are hallmarks of exciton-coupled systems, including natural chlorosomes. The CD intensity varies with each sample, even though each sample was adjusted to the same Q_y absorbance value before measurements.

**Figure 3.6:** Circular dichroism spectra for the (A) unary mixtures, (B) binary mixtures, and (C) ternary, quaternary, and quinary mixtures.
3.3.3 Time-resolved fluorescence

The quaternary and quinary mixture constructs were analyzed using picosecond time-resolved fluorescence. Both constructs were excited directly into the BChl/f Soret band at 515 nm. BChls c, d, e, f, and a minimally absorb at this wavelength. Contour plots of streak camera response, as well as representative kinetics and spectra at different delay times can be seen in Figs. 3.7 and 3.8.

Figure 3.7: Time resolved fluorescence of the quaternary biohybrid construct. (A) Contour plot of the streak camera response, (B) representative spectra taken at different delay times relative to an arbitrary time zero, (C) representative kinetics at various wavelengths on a linear scale, and (D) re-scaling of (C) on a logarithmic time scale.
Figure 3.8: Time resolved fluorescence of the quinary biohybrid construct. (A) Contour plot of the streak camera response, (B) representative spectra taken at different delay times relative to an arbitrary time zero, (C) representative kinetics at various wavelengths on a linear scale, and (D) re-scaling of (C) on a logarithmic time scale.

As mentioned in Section 3.2.5, these fluorescence spectra could not be globally fit satisfactorily using less than six monoexponentially decaying spectral-kinetic components. From analyzing the data, it is clear that there are either second-order decay components present (which
cannot be accounted for in standard global-fitting software), or so many decay pathways are present that the fitting procedure becomes too computationally taxing. In lieu of global fitting, we opted to show only representative kinetic data at various wavelengths and spectral data at different delay times relative to an arbitrary time zero ($t_0$; which is usually only calculated accurately when global fitting is successful). The representative spectra show a gradual weighting of fluorescence to longer wavelengths as delay time increases (Figs. 3.7B and 3.8B). The representative kinetics show a delayed rise of fluorescence as the wavelength becomes longer; this is especially profound in the sample containing BChl $a$ (Figs. 3.7C and 3.8C). The delay in fluorescence rise is more convincingly apparent when the kinetic plots are shown on a logarithmic time scale (Figs. 3.7D and 3.8D). The delay of fluorescence rise time implies energy transfer from high-energy pigments to low-energy pigments. In Fig. 3.8D, it is clearly shown that the fluorescence from BChl $a$ at 800 nm is delayed in rising to its maximum, meaning that BChl $a$ molecules only became excited after of the other four types of BChls in the construct becomes excited. Because the delay in rise time is on a timescale much less than 50 ps, the energy transfer processes in these constructs is ultrafast and on par with that of natural chlorosomes at room temperature.

3.3.4 Disruption of self-assembly in constructs containing BChl $a$

We compared the quaternary mixture construct (1:1:1:1 BChl $c:d:e:f$) to the quinary mixture construct (1:1:1:1:0.2 BChl $c:d:e:f:a$) via low-temperature absorption. This is shown in Fig. 3.9.
Figure 3.9: 77 K absorption spectra of the quaternary and quinary mixture constructs. The quinary spectrum is offset by 0.05 a.u., and the $Q_y$ region is magnified, to facilitate comparison.

In the quaternary mixture, the absorption spectrum in the $Q_y$ region is smooth, yet narrower than at room temperature. In the quinary mixture, the $Q_y$ region is not smooth, and fine spectral detail begins to be resolved. The may indicate that the addition of BCHl $a$ disrupts the self-assembly of the other pigments (see Discussion).
3.3.6 Comparison of the biohybrid constructs to chlorosomes that naturally contain a BChl c/d mixture

We additionally analyzed a sample of chlorosomes that naturally contain both BChl c and d, presumably mixed in a heterogeneous fashion inside each complex. A static absorption spectrum, circular dichroism, and time-resolved fluorescence streak camera image and global fitting (all at 298 K) are shown in Fig. 3.10. We specifically compare the absorption and circular dichroism spectra to our biohybrid constructs which contain a 1:1 mixture of BChl c:d. The Qy OD of the natural chlorosome sample was 1.0 in a path length of 1 cm, while the OD of the biohybrid construct was 0.2 in a path length of 1 cm. Therefore, to facilitate comparison, the CD spectrum of the biohybrid construct was scaled by a factor of 5.
Figure 3.10: The absorption (A), circular dichroism (B), and time-resolved fluorescence streak camera response (C) with globally fit spectral-kinetic components (D) of the chlorosomes from the GSB isolate which naturally contain a mixture of BChl c and d. Panels A and B additionally show a comparison to the biohybrid construct which contains BChl c and d in a 1:1 mixture.

The absorption spectrum of the natural chlorosome shows a wider Q\text{y} peak, indicating a higher level of inhomogeneous broadening. Additionally, the Soret:Q\text{y} intensity ratio is higher in the natural chlorosome than the biohybrid construct, but this can only be partially attributed to the existence of carotenoids in the natural chlorosome (see difference spectrum in Fig. 3.10A).
The CD spectra are similar from the standpoint of their conservativeness; the positive and negative portions of the spectrum in the $Q_y$ region are unequal in positive and negative intensity, respectively. Both spectra seem to indicate the presence of similarly excitonically-coupled pigments in which the transition dipole of neighboring pigments are highly aligned.

Unlike some of the biohybrid constructs, the time-resolved fluorescence of the natural BChl $c/d$ chlorosomes could be satisfactorily globally fit with a series of monoexponentially-decaying spectral-kinetic components. The noise-corrected streak camera count contour plot is shown in Fig. 3.10C and the global fit (EAFS) amplitudes are shown in Fig. 3.10D. Four major components were calculated: 10 ps, 50 ps, 85 ps, and 312 ps. The 10 ps, 50 ps, and 85 ps components likely result from energy equilibration between various pools of BChl $d$ and $c$ with subsequent transfer to the BChl $a$ in the CsmA baseplate. It is unclear how the pigments are mixed in this system; BChl $c$ and $d$ could be spatially separated within each chlorosome, but it is more likely that BChl $c$ and $d$ co-mingle within different subdomains of BChl aggregates. Finally, the 312 ps component is therefore associated with the decay of the excited state of BChl $a$ in the CsmA baseplate. This lifetime is shorter than expected for excited states of BChl $a$, which are typically closer to 1 ns.

### 3.4 Discussion

We have described here a method for the construction of biohybrid chlorosome-like light-harvesting constructs containing self-assembled natural BChls surrounded by a layer of amphipathic diblock copolymer. This facile method is scalable in size and is only limited by amount of polymer and pigment substrate. Mimicking natural photosynthetic antennas *in vitro* has been a continuous challenge. No synthetic or semi-synthetic antenna created in the lab has ever completely mimicked every beneficial property of a natural antenna. We hope that this
study signifies significant progress in the design of biohybrid or biomimetic light-harvesting molecules. Our collection of biohybrid constructs represents a systematic study of the effect of incorporating different combinations of the natural “Chlorobium chlorophylls” into single self-assembled complexes. With these results, we can begin to answer questions about why chlorosome-bearing bacteria usually only contain one type of Chlorobium chlorophyll and the self-assembly flexibility of the pigments themselves.

The biohybrid constructs containing only one type of pigment (unary mixtures) bear remarkable absorptive similarity to their natural chlorosome counterparts. Aside from lacking a CsmA-BChl a, their steady-state fluorescence emission spectra are nearly identical in shape and wavelength maxima to their natural chlorosome counterparts as well. We therefore concluded that the assembly pattern seen in these constructs is similar to what occurs in natural chlorosomes. This success emboldened us to attempt creating constructs which contain mixtures of pigment not observed in nature. Mixtures of two, three, and four different types of pigment resulted in wider absorption spectra with significantly more inhomogeneous broadening, although mixtures that incorporated BChls c and d had absorption peaks that were generally wider than those that did not. Comparison of the biohybrid construct containing a 1:1 ratio of BChl c:d to a chlorosomes from a newly discovered GSB isolate naturally containing both BChl c and d revealed that the natural chlorosome has a wider Qy absorption band as well as a higher Soret:Qy band intensity ratio. These unusual natural chlorosomes also show rapid energy equilibration among the antenna pigments with energy moving to the BChl a in the CsmA baseplate in under 100 ps from time-resolved fluorescence results.
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Altering the stoichiometry of the pigments in each construct has the beneficial effect of precisely tuning the absorption maximum and full-width-at-half-maximum (FWHM) of the Q_y and Soret peaks. This is highly encouraging from an engineering standpoint, as absorption tunability is a desirable trait for the manufacture of light-absorbing products or pigment products.

The common theme across all constructs was the general energy transfer pattern from higher energy pigment to lower energy pigment. This is especially noticeable in the ternary and quaternary constructs. After selective excitation into the Soret band of BChl f (or BChl e, if BChl f was not included), all constructs maximally fluoresced at wavelengths longer than 750 nm, signifying energy transfer to BChl d or c. This clearly indicates that each individual biohybrid antenna complex contains all of the pigment types that were added into solution during complex formation (i.e., there are not individual complexes which are homogenously BChl c only, and so on). Based on the weight of the polymer, we estimate that each polymer monolayer will be on the order of 10-15 nm thick. If two of the biohybrid complexes were in direct contact, then the pigment assemblies in each would be spatially separated by at least 20 nm; likely too far for inter-complex energy transfer to occur. Therefore, the most likely conclusion that can be drawn from the fluorescence emission profiles presented is that the pigments initially added into solution to create the biohybrid constructs are homogeneously mixed among all complexes.

The addition of BChl a to the quaternary mixture already containing BChls c, d, e, and f yielded particularly interesting results. BChl a was added to approximate the presence of the CsmA baseplate found in natural chlorosomes. In the quaternary mixture, the absorption spectrum in the Q_y region at 77 K is smooth, yet narrower than at room temperature. This indicates that each complex in solution contains all four pigments that were included during the self-assembly process and all four contribute to the inhomogenous spectral broadening involved
in self-assembly. If there were multiple populations of construct, in which there were heterogenous mixtures of different pigment combinations, then one would expect the spectrum to resolve into individual absorption peaks at cryogenic temperatures. This is clearly not the case. But, in the quinary mixture case, spectral resolving is seen in the Qy region, indicating that there are multiple populations and a general disruption of self-assembly. This is presumably caused by the fact that BChl a cannot participate in the self-assembly process, but because it is hydrophobic enough to make its way into the constructs, it must find areas to occupy, preventing the other pigments from effectively forming coordination bonds. Therefore, the BChl a is not assembled into a structure resembling the CsmA baseplate in natural chlorosomes. The BChl a molecules can only be envisioned as small “islands” in a “sea” of BChls c, d, e, and f.

Even though the BChl a cannot self-assemble like the other added BChls, it can still effectively accept energy. The time-resolved fluorescence profile (Fig 3.8) of the quinary mixture construct shows energy arriving at the BChl a starting at about 20 ps after excitation into the other antenna BChls. The lifetime of the BChl a is quite short, estimated, as the time it takes for half of the fluorescence intensity to decay, to be around 100 ps. This lifetime is considerably shorter than the lifetime of free BChl a in organic solvent or the lifetime of BChl a in the chlorosome baseplate \([1,10,20]\). It is likely that some BChl a excited states are quickly quenched by either singlet-singlet annihilation, intersystem crossing to the triplet state, or other decay pathways.

Electron microscopy was employed to generally assess the shape and size of the biohybrid constructs. The constructs are in the 150-200 nm size range along the long ellipsoidal axis, within the range commonly seen in GSB chlorosomes \([1,21,22]\). The constructs also appear
highly similar to natural chlorosomes in form. Due to experimental considerations, the interior structure of the self-assembled BChl could not be resolved. This is a future area for research.

The results shown here may give us some insight into the biogenesis pathway of natural chlorosomes. Little research has been accomplished in this area [1,23,24]. Assembly of our biohybrid constructs was driven only by gradual solvent exchange from nonpolar to polar solvent conditions in 15:1 mixtures of pigment to polymer. The ellipsoidal shape of individual constructs illustrates that the ellipsoidal shape of a whole complex is the result of BChl c/d/e/f self-assembly, no matter the mixture thereof or stoichiometry. Because pigments are unlikely to be simply released into the cytosol after biosynthesis, they are likely to be directly introduced into lipid phase. We show here that the conditions necessary for self-assembly are incredibly simple, therefore a variety of simple mechanisms may be responsible for chlorosome formation. Therefore, the following mechanisms seem plausible: (1) pigments are inserted into pre-formed lipid bodies, (2) pigments are introduced into a cellular area where lipid biosynthesis is occurring, and the meeting of the two components causes chlorosomes to spontaneously assemble, (3) pigments are injected into the space between the leaflets of the cell membrane, until the leaflet bulges and pinches off into a chlorosome. However, how the appropriate proteins localize to the chlorosome is still not well documented.

Finally, it is appropriate to ask why nature does not usually choose to place multiple types of Chlorobium chlorophyll together in the same chlorosome. Our results here show that ultrafast energy transfer is still possible when BCHls c, d, e, and f co-mingle in the same complex. The extra-wide bandwidth conferred by having all four pigments in the same chlorosome would represent a distinct advantage for a green sulfur bacterium. We believe that the reason why this phenomenon is not observed lies simply with the inability of these organisms
to silence the C-20 methylase gene \((bchU)\) and the C-7 “formylase” gene \((bciD)\). To produce all four \textit{Chlorobium} chlorophylls in the same cell would require that the \(bchU\) and \(bciD\) genes either be selectively down-regulated in their transcription, or their catalytic efficiency be reduced. Perhaps the evolutionary strategy of green sulfur bacteria has been so successful thus far that they have not needed the ability to produce all four bacteriochlorophylls in the same cell.

### 3.5 Concluding remarks

Chlorosomes represent intriguing targets for engineering; their self-assembled nature, manageable size, and high capacity for light capture are highly desired traits for those developing light-harvesting organic dyes for solar capture applications. We have successfully mimicked chlorosome pigment self-assembly, ultrastructure, and energy transfer using a mixture of natural pigments and artificial polymer. While this artificial polymer-natural pigment system is excellent for studying the natural self-assembly in photosynthetic complexes, it is likely not a desirable arrangement for consumer goods, especially in solar capture devices. It is cost-ineffective to grown green sulfur bacteria, harvest their bacteriochlorophylls, and put them into devices that function under high irradiance. These natural pigments do not have high stability in high light and aerobic conditions; these conditions exacerbate the formation of BChl triplet states which sensitize singlet oxygen. For consumer goods, those wishing to use a platform similar to this would be wise to use synthetic analogs of the natural pigments which are not subject to high amounts of intersystem crossing and can be cheaply produced \textit{en masse} in the laboratory. Using synthetic pigments with similar self-assembling ability would open further avenues for effective absorption tuning and absorption peak width, especially if the polymers were additionally functionalized such that they could be attached to surfaces. Nonetheless, this study has given us
insight into the biogenesis process of chlorosomes in vivo, while also advancing a highly flexible platform for building tunable self-assembled chromophores using pigment and diblock copolymer.

3.6 References


Chapter 3


Chapter 4: Intensity-Dependence of the Singlet and Triplet Excited States of the FMO Antenna Complex

This chapter is adapted from the previously peer-reviewed and published first-authored manuscript:


4.1 Introduction

As discussed in Chapter 1, the green sulfur bacteria and the recently discovered phototrophic Acidobacterium *Chloracidobacterium thermophilum* share not only a chlorosome antenna complex, but the Fenna–Matthews–Olson (FMO) antenna protein as well [1–5]. The chlorosome transfers energy to the FMO complex, which in turn transfers energy to the reaction center. Together, the chlorosome and FMO protein act as a funnel and wire, respectively, to aid in the excitation of the reaction center [6–8].

Within the FMO complex itself, energy transfer between individual pigments cannot be explained by semi-classical energy “hopping” mechanisms alone; there is compelling evidence that wave-like quantum coherence occurs on the femtosecond-to picosecond time scale [9–12]. The close intermolecular distances between pigments inside the complex lead to exciton
coupling in which the chromophores can be described as in a superposition of several states. These excitonic couplings and energies have been investigated both experimentally and computationally [13–16].

Structurally, the FMO protein complex is a homotrimer containing seven BChl $a$ pigments bound inside each monomer with an eighth BChl $a$ bound in a cleft between monomers, totaling 24 BChl $a$ pigments in a fully intact complex [17–20]. The FMO protein is unlike every other known light-harvesting complex in that it lacks association with carotenoids, which aid in light absorption and (B)Chl triplet state quenching. The FMO protein might be expected to be subject to increased oxidative damage in the presence of molecular oxygen, but recent evidence suggests that it is not (“The fate of triplet excitations in the Fenna-Matthews-Olson antenna protein,” manuscript submitted to *J. Phys. Chem. B* in collaboration with our group). In addition, the FMO protein displays redox-dependent energy transfer behavior: in an anaerobic environment, the protein’s fluorescence lifetimes are on the order of $\sim 2$ ns, but in aerobic environments, this lifetime shrinks to $\sim 60$ ps, indicating the presence of a redox-dependent excitation quenching pathway whose molecular mechanism is unknown [21–24].

For at least 30 years, time-resolved pump-probe spectroscopic experiments, such as transient absorption (TA), have been performed on this complex at room and cryogenic temperatures to probe behaviors of its pigments and to isolate individual pigments or groups of pigments for analysis (Figure 4.1) [13]. Through analysis of these experiments and performing our own on the FMO protein from *Chlorobaculum (Cba.) tepidum*, we have found that the laser intensity with which the pump-probe experiments are conducted has a strong effect on the distribution of contributions of excited state lifetimes of the protein-bound pigments. These experiments are similar to those performed in a previous study nearly two decades ago [25], but
we now have far better control over the photon fluxes available for experimentation. We show that when the laser intensity of a pulsed laser spectroscopic experiment is above \( \sim 10^{13} \) photons \( \times \) cm\(^{-2}\) per pulse, the reduced FMO complex behaves as if it is in an oxidizing environment, where the majority of the contribution from its 2 ns lifetime is replaced by fast (1–3 ps) singlet–singlet annihilation and the 60 ps lifetime representative of redox-dependent quenching.

**Fig. 4.1:** Steady-state near-infrared (NIR) absorption spectra of the FMO protein at 298 and 77 K, illustrating how experiments at cryogenic temperatures can separate the contributions of different groups of BChl \( \alpha \) pigments to the overall spectrum. Various calculations of site energies for the BChl \( \alpha \) molecules have produced different results; however, the general consensus is that BChls no. 4 and 3 contribute most strongly to the 816 and 825 nm absorption bands, respectively [13].

The singlet-to-triplet intersystem crossing (ISC) yield, how the triplet states evolve, and on which BChl \( \alpha \) pigments the triplet states rest are lingering questions that tie into this subject.
Studies concerning the FMO triplet state have not been revisited in well over a decade and relied on an FMO protein with a different spectral type than that from *Cba. tepidum* [13,18,26,27]. In our picosecond-to-nanosecond pump-probe TA experiments at room and cryogenic temperatures, it is possible to calculate the yield of triplet conversion of the FMO BChls. These triplet yields are also dependent on the pulse intensity with which the experiment is performed; in low photon flux conditions, there is a higher population of persistent (non-annihilating) singlet excited states that can intersystem cross to the triplet state. In conditions where the singlet–singlet annihilation effects are minimized, the apparent triplet yield was as high as $\sim 11\%$ per excitation pulse. After correcting for stimulated emission effects, we calculate the maximum triplet yield to be $\sim 27\%$. Using these data, we can begin to recommend which conditions are optimal to use to measure the FMO protein, as well as to describe how BChl *a* triplet states in the protein form and evolve on the picosecond-to-nanosecond time scale.

### 4.2 Materials and Methods

#### 4.2.1 Protein preparation

The FMO protein was purified from the green sulfur bacterium *Cba. tepidum* by a previous method [28], except that ultracentrifuged and resuspended photosynthetic membranes were used instead of whole cell lysate for carbonate treatment and protein purification. The protein was considered pure when the absorbance value at 267 nm divided by that at 371 nm was less than 0.6. The pure protein was concentrated and dialyzed back into fresh 20 mM Tris-HCl buffer (pH 8.0) (for room temperature experiments) or 20 mM Tris-HCl buffer (pH 8.0) + 60% (v/v) glycerol (for cryogenic experiments) using Amicon 30 MWCO centrifugal filters (EMD Millipore, Billerica, MA).
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FMO protein considered “oxidized” was used as-purified. FMO protein considered “reduced” was first placed into an anaerobic chamber (80% N₂, 10% H₂, 10% CO₂) and allowed to equilibrate open to the chamber for 0.5 h. Sodium dithionite (Sigma-Aldrich, St. Louis, MO) was then added to a final concentration of 10 mM to react with all remaining dissolved oxygen. After another anaerobic incubation of 0.5 h, the protein was placed into square cuvettes (10 × 10 mm) and tightly sealed before removal from the chamber for experiments.

FMO protein used in cryogenic experiments was frozen to 77 K in a VNF-100 liquid nitrogen optical cryostat (Janis Research Corp., Woburn, MA). Before and after all time-resolved spectroscopy experiments, sample integrity was verified by measuring a steady-state absorption spectrum with a UV-1800 UV/vis spectrophotometer (Shimadzu North America, Columbia, MD).

4.2.2 Femtosecond time-resolved transient absorption spectroscopy

Time-resolved pump-probe absorption experiments were carried out using Helios, a femtosecond transient absorption (TA) spectrometer (Ultrafast Systems LCC, Sarasota, FL) coupled to a femtosecond laser system described in detail previously [29]. The repetition rate of the 780 nm excitation beam was 1 kHz, consisting of a ~1 ms gap between excitation pulses. The energies of the pump beam were variably set to 0.02, 0.05, 0.06, 0.10, and 0.50 μJ per pulse in a circular spot size of 1 mm diameter, corresponding to photon intensities of (0.10 – 3.0) × 10¹⁴ photons × cm⁻² per pulse. The sample absorbance (path length 10 mm) at the Qₓ maximum, 808 nm, was 0.1. Conversions of laser pulse intensities to photon flux are shown in Table 2.1.
Table 4.1: Excitation laser (780 nm) intensities used in this study and their equivalents in photon flux.

<table>
<thead>
<tr>
<th>experiment</th>
<th>laser intensity (μJ per pulse)</th>
<th>laser photon flux (photons × cm$^{-2}$ per pulse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>0.5</td>
<td>3.0 × 10$^{14}$</td>
</tr>
<tr>
<td>TA</td>
<td>0.1</td>
<td>3.0 × 10$^{13}$</td>
</tr>
<tr>
<td>TA</td>
<td>0.06</td>
<td>1.6 × 10$^{13}$</td>
</tr>
<tr>
<td>TA</td>
<td>0.05</td>
<td>1.5 × 10$^{13}$</td>
</tr>
<tr>
<td>TA</td>
<td>0.02</td>
<td>1.0 × 10$^{13}$</td>
</tr>
<tr>
<td>TRF</td>
<td>0.0012</td>
<td>2.0 × 10$^{11}$</td>
</tr>
</tbody>
</table>

4.2.3 Picosecond time-resolved fluorescence measurements

Time-resolved fluorescence experiments were carried out using a Hamamatsu Corporation (Middlesex, NJ) universal streak camera consisting of a cooled N51716-04 streak tube, C5680 blanking unit, digital CCD camera (Orca2), and A6365-01 spectrograph from Bruker Corporation (Billerica, MA). Excitation pulses were delivered from Mai-Tai, an ultrafast Ti:Sapphire laser, generating ∼90 fs laser pulses at 780 nm with a frequency of 80 MHz consisting of a ∼12.5 ns time gap between excitation pulses into the FMO Q$_y$ absorption band. The time interval between excitation pulses was adequate to avoid buildup of the BChl $a$ triplet that will diminish observed fluorescence. (From TA experiments, we saw a low triplet yield for BChls in the FMO, which is discussed in the section 4.3.) The energy of the excitation beam was 0.0012 μJ per pulse in a circular spot of 2 mm diameter, corresponding to a photon intensity of 2 × 10$^{11}$ photons × cm$^{-2}$ per pulse. The sample absorbance (path length 10 mm) at the Q$_y$ maximum at 808 nm was 0.1. The excitation beam focus point was adjusted to be very close to the cuvette wall that was used to measure emission (at right angle), and self-absorption was practically negligible in all cases. To ensure isotropic excitation of the sample, the excitation
laser beam was depolarized (polarization was randomized) before the sample using a DPU-25 achromatic depolarizer (Thorlabs, Inc., Newton, NJ).

### 4.2.4 Transient data processing and global fitting

Group velocity dispersion of the femtosecond TA spectra was corrected using Surface Xplorer 2.0 (Ultrafast Systems LCC, Sarasota, FL) by building a dispersion correction curve from a set of initial times of transient signals obtained from single wavelength fits of the representative kinetics. Kinetic traces were then fitted to a series of exponential functions, extracting lifetimes and maximum amplitudes. If a non-decaying (“infinite”) component was necessary to properly fit the kinetic spectrum, this component was considered to reflect the conversion of the FMO protein pigments to a triplet state, which has a decay time greater than the measurement window (8 ns maximum) of the femtosecond TA system. Global fitting of the data sets was performed using a modified version of ASUfit (http://www.public.asu.edu/~laserweb/asufit/asufit.html). Global fitting was done by applying a sequential irreversible decay path model (A → B → C → D → ...). The spectral profiles obtained from this sequential fitting of the TA data sets are termed evolution-associated difference spectra (EADS) [30]. The full width at half-maximum of the instrument response function was obtained as one of the global fitting parameters and was always less than 250 fs.

### 4.2.5 BChl a purification and FMO fluorescence quantum yield determination

BChl a was purified from a BChl overproduction ΔCrtJ mutant strain of *Rhodobacter capsulatus*, which was a kind gift from Dr. Carl Bauer at Indiana University [31]. Cells were grown microaerobically in the dark in liquid RCV media for 3 days at 32 °C and harvested via
centrifugation [32]. Pigments were extracted with HPLC-grade 7:2 acetone/methanol (v/v) and transferred to a microcentrifuge tube. The solution was then centrifuged (13,000 × g for 5 min). The clear, blue-green supernatant was directly injected into an Agilent Series 1100 HPLC system (Agilent Technologies, Inc., Santa Clara, CA) equipped with a reverse-phase C-18 column (4.6 × 250 mm, Agilent Technologies, Inc., Santa Clara, CA) regulated at 20 °C. BChl a eluted at 11 min in a mobile phase of 60:36:4 acetonitrile/methanol/tetrahydrofuran (v/v) pumped at a rate of 1.5 mL min⁻¹ was collected, dried under a stream of nitrogen, and redissolved in degassed pyridine [30].

Fluorescence quantum yield measurements for FMO were performed similarly to a previous study [33], except that BChl a was the quantum yield standard and oxidized or reduced FMO were the analytes. For these measurements, aliquots of purified BChl a, oxidized FMO, or reduced FMO were transferred to square quartz cuvettes (10 mm × 10 mm) with screwtops fitted with rubber septa. Before sealing the cuvettes, the headspace was sparged with nitrogen to eliminate as much oxygen as possible. Degassed pyridine (for BChl a), 20 mm Tris-HCl buffer (for oxidized FMO), or 20 mM Tris-HCl + 10 mM sodium dithionite (for reduced FMO) was injected through the rubber septa to adjust the absorbance of the samples at their excitation wavelengths to a series of values between 0.02 and 0.10. Absorption spectra were taken after each fluorescence measurement to ensure that degradation of the pigments was negligible, as well as to record accurately the absorbance value at the excitation wavelength. Fluorescence emission spectra of BChl a and oxidized or reduced FMO at each absorbance were taken using a 602 nm (FMO) or 612 nm (BChl a) excitation beam (lamp flux ∼1 × 10¹² photons × cm⁻² × s⁻¹), measuring emission from 650–1000 nm on a customized fluorometer with an avalanche photodiode detector (Photon Technology International, Inc., Birmingham, NJ). Fully corrected
fluorescence spectra were integrated to determine quantum yield using BChl $a$ ($\Phi_F = 0.20$) as the reference [34] using the method described in [33,35]. No correction for reabsorption was made because the absorbance values were low enough that the correction values are insignificant.

Relative fluorescence quantum yield ($\Phi_F$) was solved using the following equation previously described [33,35],

$$\Phi_{F,FMO} = \Phi_{F,BChl\,a} \left( \frac{Grad_{FMO}}{Grad_{BChl\,a}} \right) \left( \frac{\eta^2_{FMO}}{\eta^2_{BChl\,a}} \right) \left( \frac{Ordinate_{1-T\,spectrum\,at\,BChl\,a\,\lambda_{ex}}}{Ordinate_{1-T\,spectrum\,at\,FMO\,\lambda_{ex}}} \right)$$  \hspace{1cm} \text{Eq. 1}

which contains a correction for an experiment using different excitation wavelengths for the standard and analyte. $Grad$ refers to the slope of a linear regression of an $x$-$y$ scatterplot of integrated fluorescence versus absorbance, and $\eta$ is the refractive index of the solvent. The $\eta$ value for pyridine is 1.51 at 20 °C (MSDS, Fisher Scientific, Waltham, MA) and 1.36 for 20 mM Tris-HCl (pH 8.0) at 20 °C (MSDS, Hampton Research, Aliso Viejo, CA)

### 4.2.6 Excitation probability analysis

To determine the probability with which multiple chromophores within a single FMO monomer will be simultaneously excited with the same pump pulse, a statistical treatment similar to a previous study was applied [36]. This model will allow us to determine if any of our laser conditions are strong enough to produce singlet-singlet annihilation effects within a single FMO monomer with any high probability. We begin by considering our laser pulse flux, $I$, in terms of photons $\times$ cm$^{-2}$ per pulse incident on our FMO sample which has an extinction coefficient, $\varepsilon$, equal to the average extinction coefficient of a single BChl $a$ in the protein ($1.53 \times 10^6$ M$^{-1} \times$ cm$^{-1}$) [37] multiplied by the number of BChl $a$ in each “interacting unit”. We define the “interacting unit” as a single monomer of the FMO protein, because we assume high levels of intramonomer energy transfer and low levels of intermonomer energy transfer due to distance
constraints. The number of BChl $a$ in a single monomer is assumed to be an average of $\sim 7.67$ in our preparations from mass spectrometry results $[17]$. Therefore, we calculate $\varepsilon_{\text{monomer}}$ to be $\sim 1.17 \times 10^7 \text{ M}^{-1} \times \text{cm}^{-1}$. The effective absorption cross-section, $\sigma$, available to the laser pulse from each FMO monomer is therefore

$$
\sigma = \frac{2303 \varepsilon}{N_A},
$$

Eq. 2

where $N_A$ is Avogadro’s number. The average number of photons simultaneously absorbed by chromophores, $x$, in the FMO monomer in a single laser pulse of intensity is given by

$$
x = I \sigma
$$

Eq. 3

It is important to note that though this average number of “hits,” $x$, can be any real number, each individual FMO monomer must absorb only an integer number of photons. If we consider $n$ to be the integer number of photons absorbed by single FMO monomers, we can produce a Poisson distribution modeling the probability distribution of simultaneous hits at each laser intensity using the following equation:

$$
P_n = \frac{x^n e^{-x}}{n!}
$$

Eq. 4

where

$$
\sum_{i=0}^{n} \frac{x^n e^{-x}}{n!} = 1
$$

Eq. 5

Therefore, $P_0 = e^{-x}$ is the fraction of FMO monomers that receive no excitations, $P_1 = xe^{-x}$ is the fraction that receives one excitation, $P_2 = (x^2/2) e^{-x}$ is the fraction that receives two excitations, and so forth. Therefore, the expression

$$
\sum_{i=2}^{n} \frac{x^n e^{-x}}{n!}
$$

Eq. 6
represents the fraction of FMO monomers that receive more than one excitation and are subject to singlet–singlet annihilation. With this distribution, the consequence of high laser intensity on the probability of singlet–singlet annihilation can be analyzed.

4.3 Results

4.3.1 Transient absorption experiments

The TA spectra taken at different delay times after excitation for each condition subjected to the FMO protein are shown in Figure 4.2. These spectra represent the raw data on which analyses were performed. It can be seen from visual inspection of these plots that the behavior of the protein at each temperature and redox condition changes as the incident laser intensity in the TA is decreased.
Fig. 4.2: The transient absorption spectra taken at different delay times after excitation for all experimental conditions performed on the FMO protein, including different incident laser intensities, different temperatures, and different redox environments.
4.3.2 Analysis of TA and TRF performed at room temperature

Figure 4.3 shows the TA kinetics, TA global analysis and TRF kinetic trace for the reduced FMO protein at 298 K. It can be seen from Fig. 4.3A that decreases in the laser intensity result in drastic changes to the TA dynamics. From fitting, the TA kinetics are composed of three to four different (~5 ps, 60 ps, 2000 ps and “infinite lifetime”) components. Decreases in laser intensity shift the contribution of these lifetimes from heavily favoring the shorter components to heavily favoring the longer components. These changes are also shown in EADS profiles (Fig. 4.3D-F), where longer lifetime components consistently show a more negative amplitude in respect to the minimum as laser intensity is decreased. We define the ~5 ps component here as mostly singlet-singlet annihilation, the ~60 ps component as the FMO’s redox-dependent quenching pathway, the ~2000 ps component as the intrinsic singlet excited state lifetime of the energetically lowest BChl a exciton(s), and the infinite component as the BChl a triplet excited state. The TRF contour plot and fluorescence dynamics in Fig. 4.3B-C indicate the fully reduced nature of this protein via the appearance of a ~2 ns lifetime [24]. A full table of fitting results from all TA and TRF experiments (including lifetimes and amplitudes) is given in Figs. 4.S1 through 4.S4.
Fig. 4.3: (A) Raw TA kinetics (circles) and their fit from global analysis (lines) for reduced FMO samples at 298 K excited with different pump laser intensities; (B) A two-dimensional contour plot of the streak camera-derived TRF data of the same sample; (C) The representative fluorescence decay trace extracted from the TRF dataset at 825 nm; (D-F) The results from global analysis of the TA datasets from each incident laser intensity using a sequential decay path (EADS). The FMO protein was excited at 780 nm in all experiments.
Figure 4.4 shows the TA kinetics, global analysis of the TA data, and TRF kinetics for the oxidized FMO protein at 298 K. Similar to the case of the reduced protein at this temperature, the decrease in laser intensity alters the distribution of contributions of different TA lifetimes. In this case, regardless of laser intensity, the TA kinetics can be satisfactorily fit without the need for an infinite component, indicating that there is no detectable population of BChl triplets. The TRF results (Fig. 4.4B-C) confirm that this protein is fluorescing in an “oxidized” regime via the appearance of only a ~60 ps lifetime [24].
Fig. 4.4: (A) Raw TA kinetics (circles) and their fits from global analysis (lines) for oxidized FMO samples at 298 K excited with different pump laser intensities; (B) A two-dimensional contour plot of the streak camera-derived TRF data of the same sample; (C) The representative fluorescence decay kinetics extracted from the TRF dataset at 825 nm; (D-F) The results from global analysis of the TA datasets from each incident laser intensity using a sequential decay path (EADS). The FMO protein was excited at 780 nm in all experiments.
Using the results from TA kinetics analysis, we are able to construct bar graphs representing the contribution of each component to the overall TA kinetics of the protein. The amplitude of each lifetime component from fitting was divided by the maximum amplitude of the TA spectrum, giving a relative amplitude, or percent-contribution of that lifetime to the system. These bar graphs are visualized in Fig. 4.5. At high laser intensities, the TA spectra are dominated by the short ~5 ps component, indicative of high levels of singlet-singlet annihilation. As the laser intensity is decreased, in the case of the reduced protein, the contribution of the 2 ns singlet excited state lifetime begins to dominate and the BChl singlet-to-triplet conversion yield increases to ~11%. In the case of the oxidized protein, the 60 ps component begins to dominate at low laser intensities and BChl conversion to triplet state is not observed.

Fig. 4.5: A bar graph representation of the contribution of individual lifetime components of the TA kinetics at 808 nm to the overall behavior in the FMO protein at 298 K in reducing (left) and oxidizing (right) conditions.
4.3.3 Analysis of TA and TRF performed at cryogenic temperatures

Figure 4.6 shows the TA kinetics, TA global analysis, and TRF kinetics for the reduced FMO protein at 77 K K. At 77 K, due to the splitting in the absorption spectrum, it is possible to separately fit the TA kinetics for the different BChl $a$ bands at 806, 816, and 825 nm. In the case of reduced FMO at 77 K, there is no qualitative difference in the TA kinetics (Figure 4.6A–C) across laser intensities. The derived lifetimes generally match those that were observed in the reduced protein at room temperature, but it is possible after global fitting (Figure 4.6D–F) to see how the differences in amplitudes of those components indicate different protein behaviors. The shape of the shortest (∼1 ps lifetime) component is drastically different between the highest laser intensity and the two lower intensities. The ∼1 ps EADS shape in Figure 4.6D is indicative of high levels of singlet–singlet annihilation, because it resembles a pure absorptive event (short lifetime with negative amplitude only), whereas the corresponding shape in Figure 4.6E,F is indicative of energy transfer or exciton diffusion (a short lifetime with an S-shaped curve) [16]; both processes occur around this time scale. The TRF spectra from Figure 4.6G,H, showing only a ∼2 ns fluorescence lifetime, confirm that this protein is indeed still “reduced” at 77 K.
Fig. 4.6: (A–C) Raw TA kinetics (circles) and their fit from global analysis (line) for reduced FMO samples at 77 K excited with different pump laser intensities. (D–F) Results from global fitting of TA data sets from each incident laser intensity using a sequential decay path (EADS). (G) Two-dimensional contour plot of the streak camera-derived TRF data of the same sample. (H) Representative fluorescence decay kinetics extracted from the TRF data set at 829 nm. The FMO protein was excited at 780 nm in all experiments.

We can apply the same bar graph representation as before for the protein in these conditions, which is represented in Figure 4.7. The effect of laser intensity is most obvious in the 825 nm absorption band, showing a distinct rise in the contribution from the singlet-excited state lifetime and triplet conversion as laser intensity is decreased. It can also be seen from these plots
that the maximum apparent triplet conversion seen is \(~10\%\) and is concentrated in the 816 nm band, and to a lesser extent, on the 825 nm absorption band.

**Figure 4.7:** Bar graph representation of the contribution of individual lifetime components from the TA kinetics at 806 (top left), 816 (top right), and 825 nm (bottom left) to the overall behavior in the FMO protein at 77 K in reducing conditions. The triplet conversion contributions are combined into a single graph (bottom right).

Figure 4.8 shows the TA kinetics, TA global analysis, and TRF kinetics for the oxidized FMO protein at 77 K. Unlike in the reducing conditions at 77 K, it is easier to see the effect of laser intensity on TA kinetics by visual inspection in Figure 4.8A–C. Upon looking at the global fitting results (Figure 4.8D–F), it is easier to quantify these differences, and again there is a large difference seen in the shape of the EADS short component between the highest laser intensity to
the two lower intensities. The TRF spectrum and kinetics in Figure 4.8G,H can be fit using two lifetimes, a medium and long component, indicating that the redox-dependent quenching in this condition is somewhat attenuated. This is reinforced by the bar graphs seen in Figure 9, which show that the amount of redox-dependent quenching (∼60 ps lifetime) is attenuated in comparison to room temperature (Figure 4.5). Also, in contrast to the oxidized protein at room temperature, the oxidized protein at 77 K must be fit using an infinite lifetime component, indicating that BChl \(a\) triplets are indeed formed in these conditions. From Figure 4.9, these triplet conversions are localized to the 825 nm absorption band only.
Fig. 4.8: (A–C) Raw TA kinetics (circles) and their fit from global analysis (line) for oxidized FMO samples at 77 K excited with different pump laser intensities. (D–F) Results from global fitting of transient absorption data sets from each incident laser intensity using a sequential decay path (EADS). (G) Two-dimensional contour plot of the streak camera-derived TRF data of the same sample. (H) Representative fluorescence decay kinetics extracted from the TRF data set at 829 nm. The FMO protein was excited at 780 nm in all experiments.
4.3.5 Fluorescence quantum yield determination

Figure 4.10 shows the fluorescence quantum yield ($\Phi_F$) determination of FMO using BChl $a$ in pyridine as a standard. The value of $\Phi_F$ in air-oxidized buffer for FMO is 0.02, and the value in dithionite-reduced buffer is 0.08. It is crucial to note that this is a static spectroscopy experiment, in which the sample is irradiated with a lamp flux of $\sim 1 \times 10^{12}$ photons cm$^{-2}$ s$^{-1}$.
Fig. 4.10: Linear correlation of integrated fluorescence vs absorbance at excitation wavelength for determination of the fluorescence quantum yield of the FMO protein in both oxidizing and reduced buffer conditions. BChl \( a \) in degassed pyridine was used as the known reference.

4.3.6 Excitation probability analysis

The results of our Poisson analysis of excitation probability in the FMO protein are shown in Figure 4.11. At the highest laser intensity, 0.5 \( \mu \)J per pulse (corresponding to \( 3.0 \times 10^{14} \) photons \( \times \) cm\(^{-2} \) per pulse), the average number of excitations in a single FMO monomer occurring due to a single laser pulse is 1.34, resulting in a broad distribution where a statistically significant probability exists in which 0, 1, 2, or 3 BChl \( a \) pigments in that single monomer can
be excited with the same laser pulse. As the laser intensity is decreased, the average number of excitations decreases sharply, approaching zero. The Poisson distributions for the other, lower laser intensities show that FMO monomers in these conditions do not have a statistically significant probability of absorbing more than one excitation in a single pulse. But, the amounts of expected singlet–singlet annihilation determined from the Poisson distributions do not exactly match the experimental transient absorption data. For example, at the 0.5 μJ per pulse level, the expected amount of singlet–singlet annihilation is about 40%, whereas the experimental data (for the reduced protein at room temperature) places this value at about 55%. A possible explanation for this is given in the section 4.4.

Fig. 4.11: Set of Poisson distributions illustrating the probability of exciting n number of pigments in a single FMO monomer with the same 780 nm laser pulse at different laser pulse intensities.
4.4 Discussion

The results presented here can begin to answer the following questions: (1) How does laser intensity distort the intrinsic decay processes in the FMO protein? (2) How do BChl $a$ triplet states evolve, and where do they rest on the picosecond-to-nanosecond time scale? (3) What do these results imply for the integrity of static spectroscopy experiments or single molecule experiments?

Addressing question (1) is fairly straightforward with the data at hand. It is clear that when the probability of exciting more than one pigment in each FMO monomer in the same pulse is high, such as 0.5 $\mu$J per pulse in our experiments, high amounts of singlet–singlet annihilation distort the shape of the overall TA kinetics. The high probability of singlet–singlet annihilation dominates and masks the longer singlet excited state components. When this singlet-singlet annihilation is minimized, the protein is able to function as expected. A study performed nearly two decades ago [25] also experimented with singlet-singlet annihilation effects in the FMO protein, but since that time the photon flux range available for experimentation has greatly improved, such that we can essentially eliminate the annihilation effects. It is possible that because the annihilation is on a similar time scale to the quantum beating behaviors seen in the FMO protein, high amounts of annihilation could perturb the observance of these phenomena. We suggest that in pulsed experiments with this protein, the use of photon fluxes less than $1 \times 10^{13}$ photons $\times$ cm$^{-2}$ per pulse ensures maximal physiologically relevant behavior. It is also interesting to note that the ability of the FMO protein to quench its excitations in an oxidizing environment is attenuated at low temperatures, regardless of laser intensity. This seems to reinforce the hypothesis from [24] that suggests the quenching mechanism in the FMO protein is chemically based and is likely due to a modified, redox-active, or radical amino acid side chain.
Addressing question (2) is also fairly straightforward, but the data here do not answer every question about FMO triplet states. From the triplet conversion rates calculated both for the protein at room temperature and the cryogenic temperature, it seems likely that triplet states do form with a significant probability when singlet–singlet annihilation effects are kept to a minimum. This occurs in low laser intensity conditions where the probability of exciting more than one BChl a per FMO monomer per pulse is very low (see Figure 4.11). This allows for a larger pool of persistent singlet excited states that have the ability to convert to triplet states. From the 77 K data shown in Figures 4.6–4.9 (specifically the EADS infinite component, which corresponds to the ground state bleaching of BChl a excitons that intersystem crossed to the triplet state), the most triplet character is seen in the 816 and 825 nm absorption bands, which, according to most site energy calculations, mostly represent BChls numbers 4 and 3. This demonstrates that the triplet state is preferentially localized on the lowest energy BChl a excitons (Figure 4.12). It is evident that the triplet state, similarly to the singlet state, is delocalized over the entire BChl a arrangement. These results also reinforce older EPR and magnetic resonance data predicting BChl number 3 as a resting place for the triplet state, though these studies used the FMO protein from Prosthecochloris aestuarii, which has a different spectral type than the protein from Cba. tepidum [18,26,27]
The triplet yields that are calculated from data in Figures 4.3, 4.4, 4.6, and 4.8 do not account for stimulated emission (SE) effects in our laser experiments. SE will deplete the initial population of excited states, eliminating their probability of intersystem crossing. To calculate the SE rate, we compared the $Q_y/Q_x$ intensity ratio from the FMO protein in TA experiments (Fig. 4.13). This ratio, at all delay times, should be identical to that in the steady state if there are no SE effects present. The $Q_x$ band is not subject to SE, as the emission from the FMO protein is
localized to the $Q_y$ transition. An increase in TA $Q_y/Q_x$ intensity ratio from that seen in the steady-state implies that extra absorption intensity is being depleted by a measurement-driven process (i.e., the probe beam). Any SE at short times after pump excitation will deplete the population of long-lived singlet excited states that are available for intersystem crossing (ISC). Since the SE signal seems to represent about 60% of the total $Q_y$ TA signal, we can expect that any apparent ISC calculated in our experiments (which are based on the amount of persistent ground state bleaching) to be only 40% of the actual that is possible if SE was not occurring. Therefore, the maximum triplet yield should be around 27% if an 11% observed triplet yield is calculated (~2.5-fold increase). This SE effect is independent of pump beam intensity and is present equally in all experiments.
Fig. 4.13: The steady-state absorption spectrum (broken line) plotted alongside the TA spectra at various delay times (solid lines) of dithionite-reduced FMO at 298 K. The TA spectra were both multiplied by -1 (to invert them for comparison) and normalized to the Qx absorption maximum (~600 nm). The pump laser fluence was 0.1 μJ per pulse for the TA experiment. The TA spectra are non-continuous between 715-760 nm due to instrument considerations.

Also, we did not observe a “building up” of triplet (infinite lifetime component) amplitude in our TA kinetics. This would have been manifested as an increase in −ΔA as experiments reached longer delay times in the 1-8 ns regime. As our TA instrumentation excites the sample at a frequency of 1 kHz (1 ms between pulses), this indicates that any BChl α triplets form and then decay on the 10−100 μs time scale. This is in agreement with the 99 μs triplet lifetime of monomer BChl α in solvent [30]. With the lack of triplet-scavenging carotenoids in
the FMO complex, it is appropriate to ask if BChl $a$ singlet-to-triplet conversion poses a danger to the bacterium, especially in the presence of oxygen. Our data, especially those shown in Figures 4.4 and 4.5, conclusively show that the FMO protein is protected from triplet formation in oxidizing conditions through the presence of redox-dependent quenching. Even if a triplet were to form on a BChl $a$ in an oxidized environment, it is possible that the energy level of that triplet state is lower than that of the energy necessary to produce singlet oxygen, thereby preventing production of a reactive species. From ref. [30], it was shown that the energy gap between fluorescence and phosphorescence for BChls was $\sim 4430$ cm$^{-1}$, on average. Using the room temperature fluorescence maximum of the FMO BChl $a$ pigments, 825 nm, the phosphorescence spectrum (indicating the triplet state energy) of the BChl $a$ pigments in FMO should have a maximum at $\sim 1300$ nm, a lower energy than that required for the production of singlet oxygen (1270 nm) [38]. Accurate in vitro measurements of the FMO phosphorescence spectrum are necessary to confirm this. If these calculations were substantiated experimentally, then the FMO protein’s redox-dependent quenching activity serves not to protect FMO itself from oxidative damage, but rather, to protect the reaction center from accepting energy in aerobic environments.

We can address question (3) by returning to Figure 4.11. By changing all pulsed units to s$^{-1}$ units and converting all photon fluxes accordingly, we can evaluate the number of excitations in a single FMO monomer per second in a static spectroscopy experiment that utilizes a lamp or static laser. At $3 \times 10^{14}$ photons $\times$ cm$^{-2} \times$ s$^{-1}$ (0.5 $\mu$J $\times$ s$^{-1}$), the average number of excitations per FMO monomer per second is 1.34, with significant probability of exciting 2, 3, or even 4 chromophores in that single second or laser shot. But, considering that 1 s is 500,000 times the maximum lifetime of the FMO BChl $a$ pigments (2 ns), the chance of two excitations in the same
monomer existing simultaneously in that time frame is minimal. Thus, in static spectroscopy experiments that use no more than $3 \times 10^{14}$ photons $\times$ s$^{-1}$ (most standard spectrophotometers and fluorometers for bulk solutions), singlet–singlet annihilation should not be an issue. In fact, even in highly sensitive single-molecule static spectroscopy experiments (e.g., that found in ref. [39]) wherein the laser fluences imply that the Poisson distribution featured in Figure 4.11 could stretch to an average excitation value of about 100 per second per protein, we expect negligible singlet–singlet annihilation. This suggests that the fluorescence quantum yields that we have determined for FMO are not being influenced by singlet–singlet annihilation effects. These relatively low quantum yield values may indicate that there is a significant amount of triplet conversion occurring in the FMO protein, or there are very few absorption events (i.e., the extinction coefficient is lower than previously determined in ref. [37]).

With respect to Figure 4.11 and the TA experimental data, it is clear that the expected amount of singlet-singlet annihilation from the Poisson distributions do not exactly match the experimental values from TA. We believe this is due to the fact that our measurements of laser power are average values and assume that the power is homogeneous across the surface area of the beam. In reality, the laser power is represented by a Gaussian curve when precisely measured across the diameter of the beam. Therefore, the laser intensity in the center of the beam will be much larger than the average intensity value. Because we aligned the beam along the side wall of the cuvettes used in experiments, this may artificially raise the local laser intensity experienced by the protein solution above the measured average beam intensity value. However, we do not believe that this inconsistency affects the integrity of our conclusions; the trends observed in our experiments are still well-defined and compelling.
Finally, it is of importance to note that as the laser power is decreased in these time-resolved experiments, there is trade-off between more physiologically relevant behavior and signal-to-noise ratio. This is clearly an instance where optimizing the signal-to-noise ratio in an experiment may be detrimental to the study of the actual function of a pigment–protein. One must also always remember that there are no assurances that isolated pigment–protein complexes should behave exactly the same way as they would in vivo. The FMO protein in vivo is not the site of primary absorption of incoming solar photons to a green sulfur bacterium; it is the chlorosome. The FMO is likely being fed by a steady stream of excitations regulated by the chlorosome. The excitation density in a single FMO protein in vivo in physiological light conditions is likely lower than what an isolated FMO protein is subjected to in an in vitro laser experiment.

### 4.5 Concluding remarks

In conclusion, we have quantified the effect of incident laser intensity on the intrinsic lifetimes and triplet state conversion in the FMO protein. We have also measured the fluorescence quantum yield of the protein. We can now suggest that using laser intensities under $1 \times 10^{13}$ photons $\times$ cm$^{-2}$ per pulse ensures the observance of maximal physiologically relevant behavior. We have demonstrated that BChl $a$ singlet-to-triplet conversion occurs with significant probability when singlet–singlet annihilation is minimized. Due to the lack of carotenoids in the protein, when BChl $a$ triplets are formed, they migrate quickly to and persist on BChls number 4 or 3, the lowest energy BChls. The triplet states that do persist are likely not energetic enough to react with molecular oxygen. With the time window of our instrumentation, determining the lifetime of the BChl $a$ triplet state is not possible, but it should be on the 10–100 μs time scale in order to avoid being gradually built up to high density in our TA experiments. This work opens
the door for further study of triplet states in the FMO protein. Further work describing the triplet states of the FMO protein involving our collaboration is described in a manuscript entitled “The fate of triplet excitations in the Fenna-Matthews-Olson complex” under review by *J. Phys. Chem. B* at the time of this thesis’ completion.

### 4.6 References


Chapter 4


### 298 K, Reduced

**Transient Absorption Spectroscopy (1 kHz pulses)**

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**Fig. 4.S1:** A table of fitting parameters for TA and TRF kinetics for the FMO protein at 298 K in a 20 mM sodium dithionite-reduced environment. *n.e.*: not evident; *n.d.:* not determined; *[A]:* raw amplitude of kinetic component; [%]: percent-contribution of kinetic component’s amplitude to total amplitude.
## 298 K, Oxidized

Transient Absorption Spectroscopy (1 kHz pulses)

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Time-Resolved Fluorescence (80 MHz Pulses)

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Fig. 4.S2: A table of fitting parameters for TA and TRF kinetics for the FMO protein at 298 K in an air-oxidized environment. \textit{n.e.}: not evident; \textit{n.d.}: not determined; \([A]\): raw amplitude of kinetic component; \([\%]\): percent-contribution of kinetic component’s amplitude to total amplitude.
**Chapter 4**

## 77 K, Reduced

**Transient Absorption Spectroscopy (1 kHz Pulses)**

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<td>inf (-0.0044)</td>
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<td>(30.7%)</td>
<td>(16.3%)</td>
<td>(42.7%)</td>
<td>(9.9%)</td>
</tr>
<tr>
<td>0.5</td>
<td>780</td>
<td>825</td>
<td>3 × 10¹⁴</td>
<td>0.7 (0.0720)</td>
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<td>(56.3%)</td>
<td>(7.5%)</td>
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<td>(4.8%)</td>
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<td>806</td>
<td>3 × 10¹⁴</td>
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<tr>
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<td>(15.0%)</td>
<td>(4.6%)</td>
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<td>1.6 × 10¹⁵</td>
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<td>(31.1%)</td>
<td>(11.6%)</td>
<td>(47.5%)</td>
<td>(9.8%)</td>
</tr>
<tr>
<td>0.06</td>
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<td>825</td>
<td>1.6 × 10¹⁵</td>
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<td>60.0 (-0.0030)</td>
<td>1998 (-0.0100)</td>
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<td>(24.1%)</td>
<td>(10.2%)</td>
<td>(57.2%)</td>
<td>(8.5%)</td>
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**Time-Resolved Fluorescence (80 MHz Pulses)**

<table>
<thead>
<tr>
<th>Laser energy (µJ)</th>
<th>Excitation (nm)</th>
<th>Flu. (nm)</th>
<th>Intensity (photons cm⁻² pulse⁻¹)</th>
<th>τ₁</th>
<th>τ₂</th>
<th>τ₃</th>
<th>τ₄ (triplet state)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0012</td>
<td>780</td>
<td>829</td>
<td>2 × 10¹⁴</td>
<td>--</td>
<td>--</td>
<td>2506</td>
<td>n.d.</td>
</tr>
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**Fig. 4.S3:** A table of fitting parameters for TA and TRF kinetics for the FMO protein at 77 K in a 20 mM sodium dithionite-reduced environment. *n.e.:* not evident; *n.d.:* not determined; [*A*]: raw amplitude of kinetic component; [%]: percent-contribution of kinetic component’s amplitude to total amplitude.
### 77 K, Oxidized

**Transient Absorption Spectroscopy (1 kHz pulses)**

<table>
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<tr>
<th>Laser energy (µJ)</th>
<th>Excitation (nm)</th>
<th>Probe (nm)</th>
<th>Intensity (photons cm⁻² pulse⁻¹)</th>
<th>$\tau_1 [A_{1}]$ [ps]</th>
<th>$\tau_2 [A_{2}]$ [ps]</th>
<th>$\tau_3 [A_{3}]$ [ps]</th>
<th>$\tau_4 [A_{4}]$ [ps] (triplet state)</th>
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</thead>
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<tr>
<td>0.5</td>
<td>780</td>
<td>806</td>
<td>$3 \times 10^{14}$</td>
<td>0.8 (-0.1477) (67.1%)</td>
<td>10.0 (-0.0626) (28.4%)</td>
<td>942 (-0.0097) (4.4%)</td>
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</tr>
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<td>0.5</td>
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<td>$3 \times 10^{14}$</td>
<td>2.6 (-0.1153) (69.6%)</td>
<td>40.0 (-0.03737) (22.6%)</td>
<td>1900 (-0.01307) (7.9%)</td>
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</tr>
<tr>
<td>0.5</td>
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<td>8.2 (-0.0516) (48.7%)</td>
<td>77.9 (-0.0300) (28.3%)</td>
<td>1204 (-0.0167) (15.8%)</td>
<td>inf (-0.0077) (7.2%)</td>
</tr>
<tr>
<td>0.1</td>
<td>780</td>
<td>806</td>
<td>$3 \times 10^{15}$</td>
<td>0.7 (-0.0213) (68.5%)</td>
<td>62.6 (-0.0051) (16.4%)</td>
<td>1109 (-0.0047) (15.0%)</td>
<td>n.e.</td>
</tr>
<tr>
<td>0.1</td>
<td>780</td>
<td>816</td>
<td>$3 \times 10^{15}$</td>
<td>1.8 (-0.0100) (27.6%)</td>
<td>100.0 (-0.0119) (32.6%)</td>
<td>1366 (-0.0145) (39.9%)</td>
<td>n.e.</td>
</tr>
<tr>
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<td>825</td>
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<td>0.7 (-0.0310) (45.1%)</td>
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<td>1400 (-0.0138) (20.1%)</td>
<td>inf (-0.0017) (2.5%)</td>
</tr>
<tr>
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<td>806</td>
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<td>0.8 (-0.0086) (67.3%)</td>
<td>91.6 (-0.0018) (13.9%)</td>
<td>1206 (-0.0024) (18.7%)</td>
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</tr>
<tr>
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<td>780</td>
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<td>134.0 (-0.0056) (44.9%)</td>
<td>1500 (-0.0039) (31.4%)</td>
<td>n.e.</td>
</tr>
<tr>
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<td>780</td>
<td>825</td>
<td>$1.5 \times 10^{15}$</td>
<td>0.8 (0.0125) (44.5%)</td>
<td>179.5 (-0.0062) (32.8%)</td>
<td>1448 (-0.0055) (19.6%)</td>
<td>inf (-0.0008) (2.8%)</td>
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</table>

### Time-Resolved Fluorescence (80 MHz Pulses)

<table>
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<tr>
<th>Laser energy (µJ)</th>
<th>Excitation (nm)</th>
<th>Flu. (nm)</th>
<th>Intensity (photons cm⁻² pulse⁻¹)</th>
<th>$\tau_1$ (ps)</th>
<th>$\tau_2$ (ps)</th>
<th>$\tau_3$ (ps)</th>
<th>$\tau_4$ (triplet state)</th>
</tr>
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<td>0.0012</td>
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<td>829</td>
<td>$2 \times 10^{13}$</td>
<td>--</td>
<td>118</td>
<td>1490</td>
<td>n.d.</td>
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**Fig. 4.S4:** A table of fitting parameters for TA and TRF kinetics for the FMO protein at 77 K in an air-oxidized environment. *n.e.:* not evident; *n.d.:* not determined; $[A]$: raw amplitude of kinetic component; [%]: percent-contribution of kinetic component’s amplitude to total amplitude.
Chapter 5: Redox-Sensitive Cysteine Residues Gate Energy Transfer in the FMO Antenna Complex

5.1 Introduction

In general, photosynthesis can be performed in the presence of oxygen (oxygenic photosynthesis, in which water is the electron source) or in the absence of oxygen (anoxygenic photosynthesis, in which other reduced species such as sulfide are the electron sources) [1–3]. Many bacteria performing anoxygenic photosynthesis contain type I reaction centers, which use Fe-S cluster chemistry to transfer electrons to ferredoxin or related molecules [4–6]. These clusters are easily damaged by molecular oxygen. Therefore, these anoxygenic phototrophs must utilize a pathway either to remove oxygen or to shut down photosynthesis whenever oxygen is encountered [1,5].

As discussed in Chapter 1, green sulfur bacteria (GSB) are anoxygenic phototrophs that contain a type I reaction center (RC). Therefore, these bacteria must avoid oxygen whenever possible. But when oxygen is encountered, the bacteria are able to decrease the output of photosynthesis effectively. This process involves creating trapping sites in the antenna complexes where de-excitation processes outcompete the rate of energy transfer to the reaction center, preventing reaction center damage. In the chlorosome, this process is fairly well-described, involving photoactive quinones dispersed amongst the BChls and Fe-S clusters
mediating the quinone redox state [7–10]. In the FMO complex, the photoprotective mechanism has thus far been more enigmatic.

Studies in the 1980’s and 1990’s first described a “dithionite effect,” in which adding the strong reductant sodium dithionite ($E_{mid}$ vs. SHE ~ -400 mV) to an oxygenated solution of FMO complex increases the complex’s fluorescence emission response and lengthens the dominant fluorescence lifetime (see Fig. 5.1) [11,12]. In oxidizing conditions, the fast lifetime (60 ps) of the quenching pathway out-competes the rate of energy transfer to the RC. In reducing conditions, this pathway effectively disappears, and the protein’s pigments fluoresce with a singlet excited state lifetime closer to that of free bacteriochlorophyll (2 ns). Those studies suggest that the quenching effect might be due to a modified aromatic amino acid, which could take a similar form to the excitation-quenching quinones found in the chlorosome. However, no such amino acid has been found via crystallography or any other method.
Figure 5.1: (A) Steady-state fluorescence emission and (B) time-resolved fluorescence kinetics at 825 nm of FMO protein in oxidizing and reducing conditions, excited at 602 nm into the Qₓ band of BChl α. In (B), the fast streak camera unit (temporal range < 1 ns) was used to capture fluorescence of the protein in oxidizing conditions (inset), while the slow streak camera unit (temporal range < 10 ns) was used for the protein in reducing conditions (main panel). Therefore the instrument response function (IRF) will appear quite different between each trace.
Chapter 5

The goals of this study are to (1) describe the molecular source of this redox-sensing effect, (2) determine the midpoint potential of the redox-sensing species, and (3) establish a viable excitation quenching mechanism based on the results. Using de novo sequencing mass spectrometry, we discovered that the two cysteine residues in each FMO monomer were highly sensitive to redox conditions. These residues are situated very close (~ 5 Å) to BChls #2 and #3, the lowest energy BChls in the complex. Permanently alkylating these cysteines in vitro drastically changed the energy transfer profile and the redox modulation of the complex. We characterized these changes by using absorption spectroscopy, circular dichroism, steady-state and time-resolved fluorescence, direct electrochemistry, and mass spectrometry. We propose that the charge state of the cysteines non-covalently affects the photophysical behavior of the BChl pigments, leading to the observed quenching effect.

5.2 Materials and Methods

5.2.1 Bacterial strains, growth conditions, and protein preparation

The green sulfur bacterium Cba. tepidum was grown and its FMO protein was isolated as previously described in Chapter 4 and other publications [13–15]. Concentrated FMO was dialyzed in 2 mL aliquots to 150 mM potassium phosphate buffer, pH 7.0, using 10 MWCO Slide-A-Lyzer MINI dialysis devices (Thermo Scientific, Waltham, MA). The higher ionic strength and neutral pH of the phosphate buffer ensures all downstream protein biochemistry does not alter the solution pH or engage in any unwanted side-reactions. All chemicals used in this study were of the highest grade and purchased from Sigma-Aldrich (St. Louis, MO).

FMO protein considered “oxidized” was used as-purified, in equilibrium with laboratory atmosphere. FMO considered “reduced” was first placed into an anaerobic chamber (95% N2, 5% H2 gases) and allowed to equilibrate open to the chamber for 0.5 h. Sodium dithionite was
then added to a final concentration of 10 mM and the solution was incubated in the chamber in the dark for an additional 0.5 hr.

5.2.2 Initial post-translational modification analysis using PEAKS

One “oxidized” FMO sample and one “reduced” FMO sample (each ~200 μg total protein) were precipitated using acetone, resuspended in urea, and digested in-solution using trypsin, similar to a previous method, except that iodoacetamide was not added to any digestion mixture after acetone precipitation [16]. For the “oxidized” sample, the procedure was performed in open air using air-equilibrated acetone. For the “reduced” sample, the procedure was performed in the anaerobic chamber using acetone that had been degassed using at least six freeze-pump-thaw cycles. Reduced samples were introduced to the air only briefly at the time of injection into a mass spectrometry instrument.

Tryptic fragments were injected into an Ultimate 3000 Nano LC system (Thermo Scientific Dionex, Sunnyvale, CA) equipped with a home-packed Michrom Magic C-18 UPLC column. Fragments were analyzed with an LTQ Orbitrap tandem mass spectrometer (Thermo Scientific, Waltham, MA). The ion source was a PicoView nano-electrospray needle (New Objective, Woburn, MA) set to the following parameters: 1.9 kV spray voltage, 200 °C capillary temperature, 80 V capillary voltage, and 100 V tube lens voltage [16]. Peptides were identified from the peptide accurate masses and product ion sequencing by either searching against the NCBI database using Mascot (Matrix Science, Boston, MA) or sequenced de novo using PEAKS (Bioinformatics Solutions Inc., Waterloo, ON). Quantitative information about the level of chemical modification in each set of tryptic peptides were obtained and compared among all samples. For additional information on the PEAKS software and methodology, see section 5.2.4.
5.2.3 Cysteine modification using NEM and dimedone

After the cysteine residues in the FMO protein were identified as biochemical targets for analysis (see section 5.3), samples of FMO protein were modified with N-ethylmaleimde (NEM), which permanently caps any free, solvent-accessible protein thiols. The modification procedure was performed as follows: a concentrated 200 μL solution (containing ~15 μM protein) of sample was reduced in the anaerobic chamber (see section 5.2.1) by adding sodium dithionite to 5 mM and incubating for 0.5 h. Presumably, any accessible cysteines in the protein would then exist in the free thiol form. The sample was then submitted to a Michael Addition by the addition of 20 μL of 125 mM NEM. The derivitization proceeded in the dark at room temperature for 2 h. The derivatized protein samples were then thoroughly buffer-exchanged into fresh air-equilibrated phosphate buffer.

Dimedone was used to cap any free protein cysteines existing as sulfenic acid. The modification procedure using dimedone was performed as follows: 1 mg of dimedone powder was added to a concentrated 200 μL solution (containing ~15 μM protein) of “oxidized” FMO sample. Incubation was performed in the dark at room temperature for 1 h. The protein samples were then thoroughly buffer-exchanged into fresh phosphate buffer.

5.2.4 Mass spectrometric confirmation of cysteine modification

After modification of cysteines as described in section 5.2.3, ~ 5 μg modified protein in 100 μL buffer was prepared for liquid chromatography and tandem mass spectrometry. The protein was precipitated using a 2-D Clean-Up Kit (GE Healthcare Biosciences, Piscataway, NJ). The protein pellets were gently dried in open air, then resuspended in 100 mM Tris-HCl, pH 8.5, with 0.1% rapigest detergent (Waters Corp., Milford, MA) and digested in solution with trypsin using a previous protocol [16]. Tryptic peptides were purified using C-18 “zip-tip” pipettes,
diluted 10x with water + 0.1% formic acid, and injected into a Q Exactive Plus hybrid quadrupole-orbitrap mass spectrometer system (Thermo Scientific, Waltham, MA) coupled to the LC system (described above in section 5.2.2) for LC-MS/MS.

The MS2 data were directly loaded into PEAKS 7.0 (Bioinformatics Solutions Inc., Waterloo, Canada) for de novo sequencing and peptide reconstruction by using an error tolerance of 10 ppm for the precursor (parent) ion and 0.01 Da for the fragment (daughter) ions. All common cysteine modifications available within the software were considered during sequencing. Peptide identification was accepted if the peptide-spectrum match’s -10lgP confidence score was higher than 30 (i.e., the chance of misidentification was unacceptably high when -10lgP < 30), the matching product-ion spectrum had more than 25% of b or y ion peaks identified, and the minimum fragment-ion relative intensity for a peptide with modification was larger than 2%.

5.2.5 Protein electrochemistry

Prior to use, the a PGE (pyrolytic graphite edge) electrode was cleaned utilizing a 1 μm alumnia slurry on cotton wool, followed by thorough sonication in water. The electrode was then passed into an anaerobic chamber (95% N₂, 5% H₂ gases, 25 °C), where 10 μL of a highly concentrated FMO solution (OD₈₀₀ nm > 30) was pipetted onto the graphite tip. After a 2 min equilibration on the electrode, the remaining protein solution was pipetted off and the PGE electrode was placed into an open glass cell containing a 75 mM mixed buffer (15 mM each of HEPES, CHES, MES, and sodium acetate, all titrated to the desired pH with concentrated NaOH or HCl) with 100 mM NaCl as a supporting electrolyte. An Ag/AgCl reference electrode (+197 mV vs. SHE) and a platinum wire counter electrode were inserted into the open glass cell. All electrodes were connected to a Model 600 Series Electrochemical Analyzer workstation (CH
Instruments, Inc., Austin, TX). At a given pH, the potential was cycled twice from +197 to -603 mV and back at a constant scan rate of 100 mV/s while measuring change in current (i.e., cyclic voltammetry using a triangular potential waveform). The second scan was retained for analysis. All data were baseline-corrected and analyzed with the program SOAS, a freely available program available at http://bip.cnrs-mrs.fr/bip06/software.html [17]. A baseline was obtained by scanning voltage with no protein adsorbed onto the electrode. Scanning to higher oxidative potential (i.e., +200 mV to +800 mV vs. SHE) did not reveal any other redox couples.

5.2.6 Steady-state optical spectroscopy and spectropolarimetry measurements

Room temperature steady-state absorption spectroscopy was performed in a UV-1800 UV/Vis/NIR spectrophotometer (Shimadzu Corp. USA, Columbia, MD). Cryogenic steady-state absorbance measurements were performed using a liquid nitrogen Optstat DN-2 optical cryostat (Oxford Instruments, Oxfordshire, UK) and a Lambda 950 Series UV/Vis/NIR spectrophotometer (Perkin-Elmer Inc., Waltham, MA). Before cryogenic experiments, pure glycerol was added to each sample to a final concentration of 50% (v/v).

All steady-state fluorescence experiments were performed using the customized PTI fluorometer (Photon Technology International Inc., Birmingham, NJ) described in Chapter 2. All FMO samples were excited into the Qx absorption band at 602 nm and emission was measured from 650 to 950 nm. Other than sodium dithionite, various other reductants were tested for their ability to affect the fluorescence of the FMO protein (see Section 5.4), including reduced glutathione, β-mercaptoethanol, sodium sulfide, dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), cysteine, sodium thiosulfate, and sodium sulfate. These reductants were all purchased from Sigma-Aldrich (St. Louis, MO) and were added to 10 mM final concentration when used.
Circular dichroism spectra were collected using a J-815 spectropolarimeter (JASCO Inc., Easton, MD), scanning from 850 nm to 650 nm. The detector sensitivity was set to 200 mdeg, sampling speed was set to 50 nm min\(^{-1}\), bandwidth set to 2 nm, and integration time set to 1 s. Four scans of each sample were signal-averaged to obtain noise correction and the averaged spectra were then manually blank-corrected using the spectrum of pure buffer.

### 5.2.7 Picosecond time-resolved fluorescence measurements

Time-resolved fluorescence measurements were carried out using the setup described in Chapter 2 with minor modification. Excitation pulses were generated at 602 nm with a final frequency of 8 MHz (125 ns between subsequent excitations). The energy of the excitation beam was less than 0.002 μJ, corresponding to a photon flux of less than 2.0 × 10\(^{11}\) photons cm\(^{-2}\) per pulse. In the streak camera, only the low speed M5677 single sweep unit was used for temporal resolution up to 8 ns.

### 5.2.8 Transient data processing and global fitting

The time-resolved fluorescence spectra were cleared from random noise by recomposing the data from the dominant principal components by using the singular value decomposition (SVD) function in SurfaceXplorer (Ultrafast Systems LLC, Sarasota, FL). Then, the spectra were globally fitted with a combination of monoexponentially decaying spectral-kinetic components convoluted by Gaussian approximation of the instrument response function (IRF). The resulting spectra are termed the decay associated fluorescence spectra (DAFS) [18]. The IRF of the streak camera tube was obtained by recording a profile of the scattered excitation laser beam in an appropriate streak camera time window.
5.3 Results

5.3.1 Initial post-translational modification search

According to our hypothesis, if an amino acid changes its chemical form depending on the redox condition of the protein, then it may be at least partially responsible for the optical changes observed in the protein. A peptide map of the initial PEAKS search for post-translational modifications (PTM’s) in oxidized or reduced wild-type Cba. tepidum FMO protein is shown in Fig. 5.2. Sequence coverage of the protein in each condition was > 98%, with only a small peptide from the N-terminal domain, VKARA, missing. The N-terminal methionine residue was also not found, consistent with common bacterial protein processing in vivo.

Because separating out real in vivo PTM’s from those caused by instrumental conditions is difficult, we focused our search not on the identity of the PTM’s, but rather on the general extent of modification on certain amino acids between the two protein samples. For example, various oxidations of tryptophan residues and methionine residues were found throughout the sequence for each sample (and these may be instrument-induced), but their relative abundance did not change appreciably between the samples. The only peptides that showed considerable change in modification extent between the two samples were one N-terminal peptide, VNAPPASPLLADCDVK (residues 36-52), and one C-terminal peptide, WVEHVCK (residues 348-354). These peptides contained the only cysteine residues (Cys49 and Cys353) in the protein, with the modification extent of the cysteines varying between protein conditions: Cys49, 20% modified in reducing conditions, 50% modified in oxidizing conditions; Cys353, 10% modified in reducing conditions, 60% modified in oxidizing conditions. The modifications found were oxidative modifications such as sulfenic acid and cysteinylation transformations. It is of
note that these two cysteine residues are spatially separated in the crystal structure by over 20 Å, precluding their ability to form disulfide bonds with one another.

**Figure 5.2:** Peptide coverage and modification identification from PEAKS for (A) FMO protein in reducing conditions, and (B) FMO protein in oxidizing conditions. The protein preparation scheme for each condition is shown above each map and the PTM identified legend is shown at right of each map. Modifications appear on these maps they represent greater than 5% of the ion intensity of that particular tryptic peptide.

From these results, we drew a tentative conclusion that the cysteine residues change their chemical form depending on the redox condition of the protein. We selected these two residues as residues of interest for further study and subjected them to *in vitro* modification using N-ethylmaleimide (NEM) and 5,5-dimethyl-1,3-cyclohexanedione (dimedone), as discussed in
Section 5.2.3. The mechanisms of NEM-addition to cysteine in the free thiol form and dimedone addition to cysteines in the sulfenic acid form are shown in Fig. 5.3.

Figure 5.3: (A) The mechanism for the addition of NEM to a cysteine free thiol, which is defined as a Michael Addition. (B) The mechanism for the addition of dimedone to a cysteine sulfenic acid, which proceeds through the enol tautomer of dimedone, a nucleophile, which adds to the cysteine sulfur atom. Water is then eliminated from the transitional complex to complete the reaction.

5.3.2 Cysteine modification

The extent of cysteine modification achieved by the addition of NEM to the FMO protein was monitored via a bottom-up proteomics approach using mass spectrometry and de novo
sequencing. From the tandem MS (product ion) data, two tryptic peptides were identified containing the cysteines of interest: VNAPPASPLLPA(DCDVK (Cys49) and WVEHECK (Cys353). NEM-derivatized cysteine was identified from product-ion spectra generated from the fragmentation of each peptide as an addition of 125.05 Da to the \( b \) and \( y \) ions attributed to the cysteine. Representative product-ion spectra for the peptide VNAPPASPLLPA(DCDVK, one showing Cys49 in the free thiol form and one showing Cys49 in NEM-derivatized form, are shown in Fig. 5.4.

![Product-ion spectra for the fragmentation of a tryptic peptide containing Cys49 from a sample of NEM-modified FMO. The top panel displays an identified peptide that contains a free thiol cysteine, while the bottom panel displays an identified peptide that contains NEM-modified cysteine.](image)

**Figure 5.4:** Product-ion spectra for the fragmentation of a tryptic peptide containing Cys49 from a sample of NEM-modified FMO. The top panel displays an identified peptide that contains a free thiol cysteine, while the bottom panel displays an identified peptide that contains NEM-modified cysteine.
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Derivatized and underivatized peptides could be found in the NEM-modified FMO sample, indicating that NEM modification was not 100% effective. A de novo sequencing approach was used to survey all types of common cysteine modifications in the sample, not just the free thiol or NEM-derivatized forms. In both the unmodified and NEM-derivatized samples, product-ion spectra were found that are consistent with Cys49 and Cys353 in the free thiol form, dehydroalanine form (elimination of sulfur atom during fragmentation), persulfide form, cysteic acid form, β-methylthiolated form, NEM-derivatized form, and NEM-derivatized form bound to one extra water molecule.

After identifying all Cys-bearing parent ion m/z ratios from the product-ion spectra, total ion intensity was calculated for each by surveying the LC and mass spectra. By determining how much ion intensity was generated for each peptide with a containing a different form of cysteine, a percent-modification extent could be calculated. The ion-intensity profiles are shown in Fig. 5.5. For Cys49, the modification extents for unmodified FMO are as follows: free thiol, 74.8%; dehydroalanine, 2.2%; cysteic acid, 9.8%; persulfide, 7.4%; β-methylthiolation, 5.8%. For Cys49, the modification extents for NEM-derivatized FMO are as follows: free thiol, 18.0%; dehydroalanine, 15.7%; cysteic acid, 24.8%; persulfide, 10.2%; β-methylthiolation, 12.7%; NEM-derivatized, 13.7%; NEM-derivatized plus one water molecule, 4.9%. For Cys353, the modification extents for unmodified FMO are as follows: free thiol, 0%; cysteic acid, 100%. For Cys353, the modification extents for NEM-derivatized FMO are as follows: free thiol, 20.7%; cysteic acid, 50.0%; NEM-derivatized, 29.3%.

No dimedone-labelled cysteines could be found in the FMO samples reacted with dimedone. Therefore, optical spectroscopy experiments on dimedone-modified protein were not performed.
Figure 5.5: Parent ion intensity profiles for the peptides VNAPPASPLLPADCDVK (Cys49, left panels) and WVEHECK (Cys353, right panels) with various cysteine modifications identified from the MS1 spectra. Note that each panel has a different y-axis scale.
5.3.3 Optical spectroscopy and spectropolarimetry of cysteine-modified protein

Upon reaction of FMO protein with NEM, changes in the some of the steady-state electronic optical spectra were observed. A panel of these spectra is shown in Fig. 5.6. Firstly, absorption spectra at 298 K showed no change, although at 77 K slight (< 2 nm) shifts are seen in the Q_y region (Fig. 5.6A with inset). This indicates that the available BCHl energy levels do not change significantly in their spacing or availability for photon absorption upon cysteine modification. However, once the protein is modified, the fluorescence emission response based on redox condition changes dramatically (Fig. 5.6B). The increase of fluorescence emission usually seen upon addition of dithionite is significantly reduced. Also, the minimum fluorescence seen when the protein is in oxidizing conditions is slightly higher in the NEM-derivatized protein vs. the unmodified protein.

Curiously, there is drastic change in the circular dichroism spectrum (Fig. 5.6C and 5.6D) of the NEM-derivatized protein even though the absorption spectrum negligibly changes. Firstly, the CD spectrum no longer is sensitive to redox condition. Secondly, the 815 nm peak in the spectrum completely disappears. This spectral change could indicate a change in the orientation of the pigment dipoles in the complex.
Figure 5.6: Various steady-state optical spectra collected for NEM-modified protein, compared with that from unmodified protein. (A) Room temperature absorption (main panel) and 77 K absorption in the Q_y region (inset), (B) Room temperature fluorescence emission in oxidized and reduced forms, (C and D) Room temperature circular dichroism in the Q_y region in oxidized and reduced conditions.

Next, we collected time-resolved fluorescence spectra on the NEM-modified FMO protein, seeking to understand why the steady-state fluorescence spectrum changes so drastically upon NEM-modification. The streak camera images, kinetics, and global fitting are shown in Fig. 5.7. The global fitting profile is noticeably different from unmodified FMO in the appearance of a new spectral-kinetic component with a lifetime of ~500 ps alongside the well-documented short-lifetime (generally ~60 ps) and long-lifetime (generally ~2 ns) components.
This 500 ps component is present in NEM-modified FMO in both oxidizing and reducing conditions. The short lifetime spectral-kinetic component is only present in the oxidized sample, and is completely converted into the long-lifetime spectral-kinetic component in the reduced sample. This corroborates the evidence from mass spectrometry (Figs. 5.4 and 5.5), which shows that not all FMO is effectively labeled by NEM. The protein sample measured by time-resolved fluorescence is clearly a mixture of NEM-modified and unmodified protein. We think that the ~500 ps spectral-kinetic component derives from one or more BChl α molecules that are electronically perturbed by the presence of NEM spatially near to their binding region within the protein.

**Figure 5.7:** (A-C) The streak camera count contour plot, kinetics at 817 nm, and global fitting profile, respectively, of NEM-modified FMO in oxidizing conditions. (D-F) The streak camera count contour plot, kinetics at 817 nm, and global fitting profile, respectively, of NEM-modified FMO in reducing conditions.
5.3.4 Electrochemistry of unmodified and cysteine-modified protein

A gallery of direct protein electrochemistry data can be seen in Fig. 5.8. Fig. 5.8.A shows the faradaic current of unmodified FMO protein at pH 7.4 when the voltage is linearly scanned from -100 mV to -400 mV vs. SHE and back again. After baseline subtraction, reductive and oxidative peaks are seen whose centers are separated by 20 mV. The separation of the peak centers is itself centered at -272 mV vs. SHE (which represents the midpoint potential of the redox process [19]). In addition, stoichiometric number of electrons in the process, \( n \), could be calculated using the following equation [19]:

\[
\delta \approx 3.53 \frac{RT}{nF}
\]

which, at 25 °C can be simplified to:

\[
\delta \approx \frac{91}{n} \text{ mV}
\]

where \( \delta \) is the full-width at half-maximum or –minimum (FWHM) of the reductive and oxidative peaks respectively, \( R \) is the ideal gas constant, \( T \) is the temperature in units Kelvin, and \( F \) is Faraday’s constant. At pH 7.41, the FWHM is 71 mV, representing an \( n \) value of 1.28. The average FWHM for FMO across all pH values measured is 69 mV, representing an \( n \) value of 1.32.

Additionally, the midpoint potential of the redox species in the FMO protein has a dependence on solution pH. Fig. 5.8B shows an example of this, with the midpoint potential shifting almost 150 mV when pH is decreased about 2 units. Fig. 5.8C shows a midpoint potential vs. pH plot for all pH values at which midpoint potential was measured. Surprisingly, this plot is linear across 4-5 pH units, with non-linear tailing occurring only below pH 3 and above pH 9. Finally, in FMO protein that has been Cysteine-alkylated with NEM, nearly all
 electrochemical activity has disappeared (Fig. 5.8D), indicating that whatever redox-active species was originally there is no longer available for redox chemistry (either it is physically blocked from incoming electrons, or it no longer can physically accept an electron).

![Diagram](image-url)

**Fig. 5.8:** Direct electrochemistry (cyclic voltammetry) of FMO protein adsorbed onto a PGE electrode. (A) The raw faradaic (solid) and baseline-corrected (dashed and inset) current of unmodified FMO protein at pH 7.4. (B) the raw faradaic current of unmodified FMO at two different pH values, showing pH dependence on $E_{\text{mid}}$. (C) A plot of $E_{\text{mid}}$ vs pH. (D) the raw faradaic (solid) and baseline-corrected (dashed) current of unmodified vs. NEM-alkylated FMO at pH 5.2.
5.4 Discussion

In this study we have revisited the molecular source of the FMO protein’s redox-dependent energy transfer modulation. An initial mass spectrometric analysis of the FMO protein showed that the two cysteine residues in the protein show different amounts of oxidative modification depending on the redox conditions of the surrounding buffer environment. We focused in on these cysteine residues, modifying them with NEM, which preferentially and permanently caps the thiol groups, preventing them from further participation in any redox chemistry. We found that the optical spectra of the protein’s pigments are altered substantially upon cysteine modification. Additionally, the electrochemical potential of the protein’s redox couple was determined to be -272 mV vs. SHE at the physiological pH of 7.4. This electrochemical potential is pH dependent and likely represents a 1:1 proton-coupled electron transfer (PCET) mechanism. The electrochemical potential is highly attenuated upon NEM-modification of the protein, providing further evidence that the electrochemical signal and optical signals are coupled together and are related to the redox cycling of the cysteine residues.

The positions of the two cysteines in each monomer are shown in Fig. 5.9. Cys49 is located on a β-strand, which ends in a β-turn that forms salt bridges with an adjacent monomer. Cys49 faces to the inside of the monomer and coordinates with the C-3 oxygen of BChl #2 at a distance of 5.0 Å. The C-3 oxygen of BChl #2 is also coordinated by the hydroxyl group of Tyr16 at a distance of 2.5 Å. Cys343 is located on an α-helix that forms salt bridges with an adjacent monomer (a different monomer than Cys49’s β-strand binds to). Cys343 also faces to the inside of the monomer and coordinates with the C-3 oxygen of BChl #3 at a distance of 4.9 Å. The C-3 oxygen of BChl #3 is also coordinated by the hydroxyl group of Ser73 at a distance of 2.8 Å. BChl #3 is considered to have has the lowest site energy in the complex; it likely solely
contributes to the lowest energy excitonic band in the complex, which localizes to 825 nm at liquid nitrogen temperature. BChl #2 is also one of the lower site-energy pigments in the complex, likely partially contributing to both the 805 and 816 nm bands at liquid nitrogen temperature [20].

Figure 5.9: A model of the FMO protein from *Cba. tepidum* (PDB: 3ENI), highlighting the environments around BChls #2 and #3 in a single monomer. Green, carbon; red, oxygen; blue, nitrogen; yellow, sulfur; tan; magnesium.

As mentioned in Chapter 1, energy transfer within the FMO complex is delocalized among the various pigments, where various pathways for energy transfer may in fact interfere with each other, giving rise to so-called “quantum coherence” effects. Although there may be many possible paths for energy to take as it transfers through the complex, transfer along some paths may be more favorable than transfer along others [20–24]. A recent report, which models energy flow through the complex *in silico*, indicated that a path from BChl #8 → #1 → #2 → #3
may be the most favorable for energy flow [25]. It makes sense from a regulatory standpoint for the protein to position reactive, regulatory amino acids near two important pigments.

The regions surrounding the two cysteines are highly conserved across green sulfur bacteria (which all contain FMO), and *Chloracidobacterium thermophilum* (the only outgroup known to contain the FMO protein). A protein sequence alignment of the FMO protein across various organisms is shown in Fig. 5.10. Cys353 is clearly retained among all members, but Cys49 is only conserved among closely-related members of the family *Chlorobiaceae* (containing the genera *Chlorobium*, *Chlorobaculum*, and *Prosthecochloris*) and genus *Chloroherpeton*. *Thermochlorobacter aerophilum* (a GSB outgroup to the family *Chlorobiaceae*) and *Chloracidobacterium thermophilum* (an outgroup to the whole phylum *Chlorobi*) contain a shifted cysteine in this region, or no cysteine at all, respectively. Interestingly, the organisms without the conserved Cys49 have the capability to grow in microaerobic conditions; conditions in which other green sulfur bacteria would not survive [26,27]. If Cys49 plays a vital role in the repression of energy transfer in FMO in oxidizing conditions, it would make sense for a microaerobe to lose this regulation in order to efficiently complete photosynthesis in the presence of oxygen. Indeed, past study on *Chloracidobacterium thermophilum* indicated that the optical properties of its FMO protein are far less responsive to addition of sodium dithionite [28].
The use of sodium dithionite in all of our experiments likely represents an ideal situation in terms of total reduction of the protein complex. Sodium dithionite is an excellent chemical reductant with an $E_{mid}$ value of $\sim -400$ mV vs. SHE, but it is not a reductant found in biology. Sodium dithionite is notoriously unstable, readily decomposing to thiosulfate and sulfite in the presence of even trace amounts of water. Therefore, sodium dithionite is not the reductant naturally acting within the cell in vivo. A previous report from our lab maintained that other reductants such as sodium borohydride and various sulfur-based reductants did not have an appreciable effect on FMO fluorescence [12]. We revisited this statement, re-testing the ability of other reductants (including more sulfur-based chemicals than before) to elicit optical changes in the protein, possibly illuminating the natural, endogenous reductant for the protein. The results are shown in Fig. 5.11.
Figure 5.11: An assay of the relative increase in fluorescence (represented by a ratio of fluorescence of the reduced protein against fluorescence of the oxidized protein, $F_{\text{red}}/F_{\text{ox}}$) conferred to the FMO protein after the addition of various chemical reductants to 20 mM final concentration. Tris(2-carboxyethyl)phosphine (TCEP) is the only reductant that is not sulfur-based. DTT: dithiothreitol; b-ME: beta-mercaptoethanol.

All reductants tested do indeed have the ability to increase the fluorescence emission of the FMO protein, although none are as effective as sodium dithionite. Even TCEP, which does not act as a reducing agent based on thiol chemistry, showed the ability to increase FMO fluorescence. Even a doubling of the fluorescence intensity in the cell is likely to result in large-
scale physiological changes, so it is possible that any one of the above reductants that are present in cells could act as the endogenous reductant for the FMO protein.

Ascribing a molecular mechanism to the redox process discovered in this study is a difficult task. The cysteines in this complex clearly are not in the proper orientation or proximity to initiate in classical disulfide chemistry. A previous study observed a 16 Gauss wide electron paramagnetic resonance (EPR) signature with a $g$ factor of 2.005 in oxidized samples at 77 K [29]. This signature disappeared upon chemical reduction of dithionite. The previous study attributed these signatures to aromatic free radicals such as semiquinones or tyrosyl radicals. However, in light of the new data presented here, as well as in-depth studies of sulfur-based radicals [30–33], the EPR signature could also result from a cysteine thiyl radical. Because the signature only appears at cryogenic temperatures, it is not possible to rule out that it is only an artifact of low temperatures (i.e., the radical is not stable at room or physiological temperature). A 1:1 PCET process implicated from electrochemistry certainly fits with a radical type mechanism where the oxidized form of the protein contains a cysteine thiyl radical, while the reduced protein contains a cysteine free thiol. At the time of writing this thesis, only preliminary EPR work has been completed on the protein, with future EPR experiments planned specifically for the NEM-derivatized protein. If the radical signature disappears upon the derivitazation of cysteines with NEM, then this would serve as evidence for the radical being localized on the cysteines.

The mass spectrometry analysis using de novo sequencing also showed a significant amount of cysteine modification to cysteic acid (Cys-SH $\rightarrow$ Cys-SOH). Cysteic acid modification is possible through the electrospray process while injecting the sample into the mass spectrometer, but the relative amount of cysteic acid modification does vary based on the
redox condition of the protein (see Fig. 5.2 and 5.5). While we could not find any labelled FMO peptide after adding dimedone (which specifically labels Cys-SOH), this does not imply that cysteic acid does not exist in vivo. Cysteic acid is commonly observed in proteins which are subjected to ROS. Perhaps the introduction of oxygen into the protein when subjected to oxidizing conditions creates a ROS which attacks the cysteine residue, changing its redox state. However, a transformation from cysteine to cysteic acid requires more electrons than is consistent with the electrochemistry data shown here.

We do not think that the cysteine residues actually covalently bind to any of the BChl pigments in the complex when undergoing their redox transformation. A Cys-BChl covalent interaction would change the absorption spectrum of the complex; this type of change is not observed. The observation of a ~500 ps fluorescence component from TRF in NEM-derivatized protein suggests that the change in electron density or size of the cysteine side group alters the electronic environment around the pigments, changing the energies or the coupling of their excited states. Due to the precise orientation of the BChl pigments in the protein, the electronic dipole moments of the pigments are aligned, conferring ultrafast energy transfer behavior. The interruption of this precise orientation (via electrostatic changes around the pigments) has significant effects on the energy transfer behavior of the pigments.

Our working hypothesis is that the redox-modulation process in the FMO complex is mediated by thyl radicals that engage in an electron transfer-recombination mechanism to de-excite the BChls in the complex. In this mechanism, an optically-excited BChl pigment in close proximity to a thyl radical cation becomes a good reducing agent, resulting in a neutral thiol and a ground state BChl radical cation. The BChl radical cation is then a good oxidizing agent, removing one electron from the thiol, resulting in a thyl radical and ground state BChl. In this
scenario, the excitation energy the BChl originally possessed is released as heat. Dithionite may indeed be an unusually good reducing agent for the cysteine thiyl radical (if it exists) because it itself is in equilibrium with \([\text{SO}_2]^\cdot\) radical in solution [34], allowing for radical termination to occur.

We think that the cysteine-dependent redox-dependent quenching mechanism in the FMO protein is unique to known photosynthesis. The FMO protein is unique among photosynthetic antennas in that it does not contain photoprotective carotenoids, which normally quench excited states and prevent singlet oxygen sensitization. With evidence emerging that the energy level of triplet excited state of the FMO protein is too low to sensitize singlet oxygen [35], it is appropriate to ask why the FMO protein needs a quenching mechanism in the first place. The answer seems to lie with the susceptibility of the reaction center to oxidative damage in the presence of oxygen. The FMO protein likely contains this redox-dependent quenching mechanism not to protect itself from damage, but to protect the reaction center from receiving excitations in oxidizing conditions.

5.5 Concluding remarks

In conclusion, we have used mass spectrometry, chemical labelling, optical spectroscopy, and electrochemistry to implicate cysteine residues as responsible for the FMO protein’s redox-dependent quenching mechanism. It is our current hypothesis that the redox process in the FMO protein is mediated by thiyl radicals present on the cysteines in the protein in oxidizing conditions, which are transformed into free thiols upon chemical reduction. A plausible mechanism includes optically excited BChl molecules de-exciting via an electron transfer-recombination mechanism with a cysteine thiyl radical, where the excitation energy is dissipated as heat. This de-excitation mechanism is similar to that postulated in chlorosome antennas,
although there the redox-modulated species is a quinone molecule. More in-depth measurement of the EPR signatures in the organic radical region \( g \sim 2.000 \) are planned, especially in the NEM-modified sample.

### 5.6 References


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Chapter 6: Conclusions and Future Directions

6.1 The Importance of Photosynthesis Research

As the Earth’s fossil fuel reserves gradually dwindle and the negative environmental impact from their mining mounts, our human society must increasingly rely on carbon-neutral fuel sources. Recent estimates calculate that if global atmospheric warming is to be limited to an average 2 °C rise over pre-industrial times, cumulative carbon emissions between the years 2011 to 2050 must be capped at 1.1 teratonnes CO$_2$ [1–3]. This represents about one-third of the available fossil fuel reserves, and illustrates that unabated fossil fuel use could spell disaster for the global environment, economy, and security [3,4]. These calculations further suggest that unabated fossil fuel use could eliminate all ultimately-recoverable fossil fuel resources, even those that are currently undiscovered, on Earth before the year 2200 [5].

Solar energy capture, in the form of biomass, biodiesel, water-splitting catalysis, or direct current, has shown promise as an alternative energy source and food source. Solar energy capture is ultimately inspired by, or directly utilizes, the biological process of photosynthesis. It is, therefore, a crucial time in the study of photosynthesis. As we gain a better understanding of photosynthetic organisms and how they capture and store energy, we gain better perspectives on how to capture and store solar energy for humanity’s use. As photosynthesis scientists, it is our task to study the diversity of photosynthetic organisms and structures, elucidating irreducible aspects of the process that could eventually aid in the development of next-generation solar capture technology and more efficacious crops for agricultural food production.
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Some members of the photosynthesis scientific community have been interested in small photosynthetic microbes called Green Sulfur Bacteria (GSB). Their photosynthetic antennas allow these microbes to capture light efficiently at the extremes of low photon flux, yet are remarkably simple biochemically [6–9]. The chlorosome antenna offers lessons in the adaptive absorptive power of self-assembling porphyrin-based pigments, while the Fenna-Matthews-Olson (FMO) protein complex reveals the secrets of biological quantum coherence and the basic physics of the coupling between a chromophore and the protein environment in which it exists. The research within this thesis advances our understanding of the basic photophysical and biochemical processes guiding the efficient light-harvesting in these antennas.

6.2 The Chlorosome Antenna Complex

The production of a green sulfur bacteria mutant containing BChl f allowed us a unique opportunity to study a previously unknown chlorophyll. We were able to analyze its excited state behavior in both monomer and oligomer (within chlorosomes) forms, showing that BChl f is fundamentally similar to BChl e and Chl b, each of which also contain a formyl substituent at the C-7 position on the chlorin ring. We also were able to show that BChl f oligomers transfer energy to the CsmA-BChl a baseplate complex with a lower efficiency compared to that of BChl e oligomers and to as suggest that this decrease in efficiency is mostly due to decreased spectral overlap instead of other structural or assembly-related factors. Obviously, it would be a significant discovery to find a naturally-occurring BChl f-containing green sulfur bacterium. These organisms, if they exist, probably live in high-light anoxic niches devoid of the filtering effects of Chl a, Chl b, or Chl d.

Because we are one of the only research groups in the world with access to the full set of “Chlorobium chlorophylls” (BChls c, d, e, and f), we became interested in simulating BChl self-
assembly \textit{in vitro}. We successfully created chlorosome-like biohybrid structures using natural BChl c, d, e, f, and a mixed with amphiphilic diblock copolymer. These structures look just like natural chlorosomes in form wherein the aggregated BChl is surrounded by a diblock copolymer layer that mimics the natural lipid monolayer. Future work in this area could be devoted to expanding the repertoire of synthetic chromophores that could substitute for the naturally-derived BChls. Expanding this pallet of chromophores would allow for precise tuning of absorption and energy transfer properties beyond what is currently available in nature. It is probably unwise from a manufacturing standpoint to develop self-assembling light-harvesting dyes for solar capture applications, or otherwise, using natural pigments only.

Additional study into the CsmA-BChl a baseplate complex is also warranted. There is still no consensus on how the small CsmA proteins oligomerizes and incorporate BChl a. It is currently believed that the sole histidine residue in each CsmA protein coordinates to the central Mg atom of the BChl a, but the mechanism for interaction of adjoining CsmA proteins to produce a paracrystalline structure is still under debate [10–12].

Although the chlorosome’s assembly mechanism is mostly unknown, there are two current hypotheses on chlorosome bio-assembly in the literature. One hypothesis suggests that the chlorosome is a specialized lipid body requiring no special cellular machinery to form, with the driving force behind biogenesis being the self-assembly of BChl inside of the cell membrane. As this accumulation grows, proteins become localized to the growing bulge, and the cytosolic membrane leaflet buds out until it pinches off into the chlorosome [13]. This hypothesis is bolstered by the observations of horizontal gene transfer of essential photosynthetic proteins and low homology of non-essential components between phyla containing chlorosomes. The other hypothesis involves biogenesis starting with synthesis of the CsmA baseplate. This baseplate
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apparatus then attaches to the FMO protein and aggregates free lipids, BChl, and proteins around itself, slowly building up the chlorosome [11]. This hypothesis is reinforced by observations that chlorosome proteins are not found elsewhere in the cell and that the lipid composition of the chlorosome is different than that of the whole cell. We think this research area deserves more attention, since knowledge of nature’s most effective method of building these large systems may aid efforts in constructing biohybrid complexes like those we have constructed in Chapter 3.

The evolution of the chlorosome and its proliferation among seemingly disparate phyla is also an interesting point. Whereas the exact sequence of events is unknown, it is likely that horizontal gene transfer for the essential BChl biosynthesis and chlorosome-specific genes occurred between Chlorobea and the other groups of green bacteria, perhaps multiple times [13]. It is currently thought that the chlorosome is one of the earliest-evolving antenna complexes owing to its simplicity, and this most likely occurred in the ancestor of the Chlorobea. Parsing together an evolutionary profile of green sulfur bacteria and their horizontal gene transfer to other phyla would help to explain under what circumstances nature prefers the simplicity of pigment-pigment interactions for light harvesting over more complicated pigment-protein interactions.

6.3 The FMO Antenna Protein

The work described in this thesis advances our knowledge substantially about the FMO antenna protein, which serves a model protein for the study of chromophore-protein interactions. We have demonstrated conditions in which spectroscopic study of the protein can actually induce non-physiologically relevant activity (singlet-singlet annihilation) and successfully calculated intersystem crossing yields for the protein after initial excitation. Work with Prof. Sergei Savikin’s group at Purdue University extended our ultrafast laser work into the μs time-
range, allowing us to actually determine the excited triplet state lifetime of FMO. This work has been submitted to *J. Phys. Chem. B* at the time of writing this thesis.

We have also finally shed light on a phenomenon first seen thirty years ago in the FMO protein, but since has never been solved: its redox-dependent excitation quenching activity [14,15]. Through mass spectrometric analysis, cysteine labelling, electrochemistry, and optical spectroscopy, we have determined that the cysteine residues in the FMO protein are sensitive to the redox condition of the protein and somehow are able to modulate the excited state lifetimes of the BChl *a* pigments in close proximity. Future work in this area would involve the devising of a discrete molecular mechanism of this effect and corresponding calculations on how the redox states of the cysteines would affect the excited states of nearby pigment molecules. Additionally, this mechanism can be tested in the FMO protein from other organisms, specifically those from the acidobacterium *Chloracidobacterium thermophilum*, and the newly discovered semi-aerobic green sulfur bacterium *Thermochlorobacter aerophilum* [16,17]. These two FMO-containing organisms, discovered by Prof. Don Bryant at The Pennsylvania State University, seem to live in more oxidizing conditions than all other known FMO-containing organisms, so it would be interesting to see how their FMO protein responds to those conditions. Also, a new FMO mutagenesis system pioneered by Dr. Rafael Saer in our group is poised to greatly enhance our understanding of not only FMO’s redox control mechanism, but nearly every other aspect of the system, including the precise role of each individual pigment.

Of course, more highly refined structural data for this protein complex would aid in the above efforts, as well as in the efforts by others who require structural data for various quantum mechanical calculations of energy transfer behavior. Our group has collaborated with Oak Ridge National Laboratory to produce a high-resolution neutron diffraction crystal structure of the
FMO protein. Such a structure would greatly enhance our knowledge of the FMO protein’s structure. This type of structure promises enhanced positioning of atomic nuclei in the three-dimensional structure, as well as discrete positioning of hydrogen atoms, which can only be inferred via X-ray diffraction crystallography, yet offer enormous amounts of information about protein environment and protonation states [18]. Our efforts in this area seem to be coming to fruition: the completion of this neutron diffraction structure should come soon after the completion of this thesis.

6.4 Final Thoughts

The continuing study of green sulfur bacteria, and photosynthesis in general, is poised to open up new avenues for the development of carbon-neutral energy sources. We seem to be continually finding new exciting ways in which these organisms adapt to new biotic and abiotic conditions. Self-assembling chromophores may hold the key to producing next-generation solar capturing devices. Our work here demonstrates that we can both mimic and improve on nature’s self-assembly methods. Additionally, our work with the FMO protein shows that there are clearly many diverse ways in which light-harvesting mechanisms can be regulated at the atomic level. We also look forward to the publication of our neutron diffraction crystal structure of the FMO protein; this will be the first structure published of its kind in the area of photosynthesis and will greatly aid all who wish to study the quantum nature of energy transfer at the atomic level.
6.5 References


Title: Structural Variability in Wild-Type and bchQ bchR Mutant Chlorosomes of the Green Sulfur Bacterium Chlorobaculum tepidum

Author: Swapna Ganapathy, Gert T. Oostergetel, Michael Reus, et al

Publication: Biochemistry
Publisher: American Chemical Society
Date: Jun 1, 2012

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Author: Richard Y-C. Huang, Jianzhong Wen, Robert E. Blankenship, et al

Publication: Biochemistry
Publisher: American Chemical Society
Date: Jan 1, 2012

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Title: The Eighth Bacteriochlorophyll Completes the Excitation Energy Funnel in the FMO Protein

Author: Marcel Schmidt am Busch, Frank Müh, Mohamed El-Amine Madjet, et al

Publication: Journal of Physical Chemistry Letters

Publisher: American Chemical Society

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2. **G.S. Orf**, C.L. McIntosh, H. Zhang, D.M. Niedzwiedzki, and R.E. Blankenship, Reactive cysteine residues gate energy transfer in the FMO complex from *Chlorobaculum tepidum*.
3. G. He, D.N. Niedzwiedzki, **G.S. Orf**, and R.E. Blankenship., Energy transfer kinetics between the FMO and reaction center core complexes in the green sulfur bacterium *Chlorobaculum tepidum*.

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Bacteriochlorophyll f: properties of chlorosomes containing the “forbidden chlorophyll”

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INTRODUCTION
Chlorosomes are the defining property of green bacteria and are the light-harvesting structures used for phototrophic growth of these bacteria (Blankenship and Matsuura, 2003; Frigaard and Bryant, 2006; Oostergetel et al., 2010; Bryant et al., 2012). Green bacteria include all known phototrophic members of the eubacterial phylum Chlorobi, some members of the Chloroflexi, and “Candidatus Chloracidobacterium thermophilum,” the only known phototrophic member of the phylum Acidobacteria (Bryant et al., 2007, 2012). Green sulfur bacteria (GSB) that are green in color produce chlorosomes containing either bacteriochlorophyll (BChl) d or BChl c and the carotenoid chlorobactene, but brown-colored GSB produce chlorosomes containing BChl e and usually the carotenoid isorenieratene (Chew and Bryant, 2007; Maresca et al., 2008; Liu and Bryant, 2012). A single chlorosome can contain up to ∼250,000 BChl c, d, or e molecules (Martinez-Planells et al., 2002; Montaño et al., 2003) which self-assemble into one of several different suprastructure (Ganapathy et al., 2009, 2012; Garcia Costas et al., 2011). A GSB cell contains ∼200 chlorosomes, and thus a green bacterial cell contains ∼50 million BChl molecules, which together account for ∼30% of the cellular carbon (Frigaard and Bryant, 2006). These enormous light-harvesting antennas allow green bacteria to grow at extremely low irradiances at which no other phototrophs can survive. Examples include GSB that grow at a depth of ∼110 meters in the Black Sea (Manske et al., 2005; Marschall et al., 2010) and a GSB that was isolated at a depth of ∼2200 m on the floor of the Pacific Ocean near a black smoker (Beatty et al., 2005).

The chlorophylls (Chls) found in chlorosomes were once commonly referred to as “Chlorobium” Chls, and they differ from other (bacterio)chlorophylls [(B)Chls] in several important ways (Chew and Bryant, 2007; Liu and Bryant, 2012). Firstly, although these molecules are commonly referred to as BChls, they are in fact chlorins and have properties more similar to Chl a than to those of bacteriochlorins, such as BChl a. Secondly, they carry a hydroxyl group at the chiral C-3′ carbon atom, and they lack the methylcarboxyl moiety found in all other types of (B)Chls at C-13′. These two properties allow BChl c, d, and e to self-aggregate in a protein-independent manner in the interior of the chlorosome (Ganapathy et al., 2009, 2012). Thirdly, these BChls can be methylated at any or all of three positions, C-8, C-12, and C-20, on the periphery of the tetrapyrrole macrocycle (Maresca et al., 2011).
Characterization and deposition of various light-harvesting antenna complexes by electrospray atomization

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Abstract Photosynthetic organisms have light-harvesting complexes that absorb and transfer energy efficiently to reaction centers. Light-harvesting complexes (LHCs) have received increased attention in order to understand the natural photosynthetic process and also to utilize their unique properties in fabricating efficient artificial and bio-hybrid devices to capture solar energy. In this work, LHCs with different architectures, sizes, and absorption spectra, such as chlorosomes, Fenna–Matthews–Olson (FMO) protein, LH2 complex, and phycobilisome have been characterized by an electrospray-scanning mobility particle-sizer system (ES-SMPS). The size measured by ES-SMPS for FMO, chlorosomes, LH2 complex, and phycobilisome were 6.4, 23.3, 9.5, and 33.4 nm, respectively. These size measurements were compared with values measured by dynamic light scattering and those reported in the literature. These complexes were deposited onto a transparent substrate by electrospray deposition. Absorption and fluorescence spectra of the deposited LHCs were measured. It was observed that the LHCs have light absorption and fluorescence spectra similar to that in solution, demonstrating the viability of the process.

Keywords Electrospray-scanning mobility particle sizer (ES-SMPS) · Electrospray deposition · Light-harvesting complexes (LHCs)

Introduction

Solar energy harvesting is receiving considerable attention because it is a carbon neutral and a renewable source of energy. Photosynthetic organisms have evolved over billions of years to harvest this energy efficiently. Light-harvesting complexes (LHCs), present in photosynthetic organisms, are key to absorption of sunlight and converting it to chemical energy. The LHCs are being studied to understand the process of photosynthesis and to utilize them in making useful bio-hybrid and artificial devices to harvest solar energy [1–6]. The light-harvesting complexes contain systematically arranged dye molecules to maximize light absorption. The absorbed light is converted to excitons and transferred to the reaction center by energy transfer processes. In the reaction center, the exciton undergoes charge separation, which drives biochemical processes. Photosystem II (PSII), from cyanobacteria and plants, has been used for making bio-sensors to detect pollutants [1]. Reaction centers such as PSII [2], bacteriorhodopsin [3], Photosystem I [4], and light-harvesting antenna complexes or their parts such as C-phycocyanin [5] and chlorosomes [6] have been used in fabricating bio-hybrid solar devices.

LHCs need to be characterized in order to understand their functionality and fabricate a biohybrid device. Since LHCs from different organisms have different architectures, they have different sizes, shapes, and absorption spectra.
Spectroscopic insights into the decreased efficiency of chlorosomes containing bacteriochlorophyll

Gregory S. Orf, Marcus Tank, Kajetan Vogl, Dariusz M. Niedzwiedzki, Donald A. Bryant, Robert E. Blankenship

Abstract

Chlorosomes are light-harvesting antenna complexes that occur in green photosynthetic bacteria which have only been shown naturally to contain bacteriochlorophyll (BChl) c, d, or e as the principal light-harvesting pigments. BChl f has long been thought to be an obvious fourth member of the so-called Chlorobium chlorophylls, because it possesses a C-7 formyl group like BChl e and lacks a methyl group at C-20 like BChl d. In organisms that synthesize BChl c or e, the bchU gene product encodes the enzyme that methylates the C-20 position of these molecules. A bchU null mutant of the green sulfur bacterium Chlorobaculum limnaeum strain 1677, which normally synthesizes BChl e, has recently been generated via insertional inactivation, and it produces chlorosomes containing BChl f [Vogl et al., 2012]. In this study, chlorosomes containing BChl f and monomeric BChl f in pyridine were characterized using a variety of spectroscopic techniques, including fluorescence emission and excitation spectroscopy, fluorescence lifetime and quantum yield determinations, and circular dichroism. These spectroscopic measurements, as well as Gaussian simulation of the data, show that chlorosomes containing BChl f are less efficient in energy transfer than those with BChl e. This can primarily be attributed to the decreased spectral overlap between the oligomeric BChl f (energy donor) fluorescence emission and the BChl e (energy acceptor) absorption in the chlorosome baseplate. This study allows us to hypothesize that, if they exist in nature, BChl f-containing organisms most likely live in rare high-light, anoxic conditions devoid of Chl a, d, or BChl e filtering.

1. Introduction

Chlorosomes are the distinctive light-harvesting antenna complexes of green bacteria, including all phototrophic members of the green sulfur bacterial (GSB) phylum Chlorobi, some members of the Chloroflexi, and “Candidatus Chloracidobacterium thermophilum,” the only known chlorophototrophic species of the Acidobacteria [1–3]. These large antenna complexes are optimized to operate efficiently at extremely low photon fluxes, which allow these organisms to grow at the lowest irradiance levels recorded for photosynthetic growth [2,4]. In members of the Chlorobi and “Ca. C. thermophilum,” the chlorosome funnels the excitation energy through the baseplate to the Fenna-Matthews-Olson (FMO) protein and then to the reaction center, where the excitation energy is converted to chemical energy. Chlorosomes are sacs filled with pigments, in which a lipid monolayer envelope, which is interspersed with proteins, surrounds the self-assembled, light-harvesting bacteriochlorophylls (BChls). Single chlorosomes contain between 100,000 and 250,000 BChl c, d, or e molecules, depending on species [5,6]. The self-assembly of the BChl pigments is largely independent of protein influence and several oligomeric structural patterns have been identified [6–11]. Carotenoids and quinones, which aid in light absorption, triplet state quenching, and redox control, are also dispersed throughout the interior of the chlorosome [3,12–15]. The self-assembled BChls transfer excitation energy to the CsmA baseplate, a paracrystalline BChl α-protein complex integrated into the lipid monolayer of the chlorosome, which serves as the interface to the FMO protein [16–18].

Bacteriochlorophylls c, d, and e share a common basic structure but differ specifically at their C-7 and C-20 substituents, which
Chlorosome antenna complexes from green photosynthetic bacteria

Gregory S. Orf · Robert E. Blankenship

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Abstract Chlorosomes are the distinguishing light-harvesting antenna complexes that are found in green photosynthetic bacteria. They contain bacteriochlorophyll (BChl) c, d, e in natural organisms, and recently through mutation, BChl f, as their principal light-harvesting pigments. In chlorosomes, these pigments self-assemble into large supramolecular structures that are enclosed inside a lipid monolayer to form an ellipsoid. The pigment assembly is dictated mostly by pigment–pigment interactions as opposed to protein–pigment interactions. On the bottom face of the chlorosome, the CsmA protein aggregates into a paracrystalline baseplate with BChl a, and serves as the interface to the next energy acceptor in the system. The exceptional light-harvesting ability at very low light conditions of chlorosomes has made them an attractive subject of study for both basic and applied science. This review, incorporating recent advancements, considers several important aspects of chlorosomes: pigment biosynthesis, organization of pigments and proteins, spectroscopic properties, and applications to bio-hybrid and bio-inspired devices.

Keywords Chlorosome · Green bacteria · Bacteriochlorophyll · Light-harvesting complex · Bio-hybrid solar cells

Introduction

Photosynthesis, the metabolic process that converts light energy into chemical energy, is accomplished via complexes containing pigments. Reaction center pigment-containing proteins use light energy to move electrons between molecules against their redox potential. Reaction centers are membrane proteins, and in many cases have low absorption cross-sections. Large light-harvesting antenna complexes associate with reaction centers to increase the absorption cross-section, while also regulating the flow of absorbed energy (Blankenship 2002; Chen and Scheer 2013). There are a myriad of light-harvesting antenna structures, all tuned to maximize their particular organism’s metabolic effectiveness in their own photic environment.

Green bacteria, including the green sulfur bacteria (GSB) Chlorobi, the filamentous anoxygenic phototrophs (FAP) Chloroflexi, and the phototrophic Acidobacteria “Candidatus Chloracidobacterium”, contain a light-harvesting complex called the chlorosome (Blankenship and Matsuura 2003; Bryant et al. 2007; Cohen-Bazire et al. 1964; Oostergetel et al. 2010). The green bacteria, especially the Chlorobi, photosynthesize efficiently at the extremes of low photon flux. Some species of the Chlorobi are adept at photosynthesizing using only the infrared radiation given off by deep sea thermal vents (Beatty et al. 2005) or leftover solar radiation at the bottom of the photic zone in a stratified lake (Manske et al. 2005; Marschall et al. 2010). In these extreme cases, population doubling times may be on the order of years.

To achieve this, the light-harvesting complex must not waste any incident energy through competing relaxation or quenching processes. The chlorosome allows for this efficient light absorption via its unique structure composed of an ellipsoidal sac surrounded by a lipid monolayer and
Photophysical Properties of the Excited States of Bacteriochlorophyll f in Solvents and in Chlorosomes

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ABSTRACT: Bacteriochlorophyll f (BChl f) is a photosynthetic pigment predicted nearly 40 years ago as a fourth potential member of the Chlorobium chlorophyll family (BChl c, d, and e). However, this pigment still has not been found in a naturally occurring organism. BChl c, d, and e are utilized by anoxygenic green photosynthetic bacteria for assembly of chlorosomes—large light-harvesting complexes that allow those organisms to survive in habitats with extremely low light intensities. Recently, using genetic methods on two different strains of Chlorobaculum limnaeum that naturally produce BChl e, two research groups produced mutants that synthesize BChl f and assemble it into chlorosomes. In this study, we present detailed investigations on spectral and dynamic characteristics of singlet excited and triplet states of BChl f with the application of ultrafast time-resolved absorption and fluorescence spectroscopies. The studies were performed on isolated BChl f in various solvents, at different temperatures, and on BChl f-containing chlorosomes in order to uncover any unusual or unfavorable properties that stand behind the lack of appearance of this pigment in natural environments.

INTRODUCTION

Chlorophylls and bacteriochlorophylls ((B)Chls) are the most abundant natural photosynthetic pigments employed in several important roles in the process of photosynthesis, including light absorption, primary charge separation in reaction centers, and excitation transfer.1,2 The basic spectroscopic properties of (B)Chl’s can be deduced from the “four-orbital” model of Gouterman.3,4 The model assumes that the major bands visible in the spectrum of electronic absorption are associated with $\pi \rightarrow \pi^*$ electronic transitions that derive from electron promotions involving the two highest occupied molecular orbitals (HOMOs) and the two lowest unoccupied molecular orbitals (LUMOs). The resulting electronic transitions are polarized along either the $\chi$- or $\gamma$-axis of the macrocycle (Figure 1) and are commonly referred to as the $Q_x$, $Q_y$, $B_x$, and $B_y$ bands.3–6 The $B$ transitions appearing in the UV (350–470 nm) range typically strongly overlap and are usually described as the Soret band. The $Q_x$ band can span the spectral region from 600 to 800 nm, depending on the degree of macrocycle saturation and the presence of specific side chain modifications. The $Q_y$ band appears in the 500–600 nm range, is the least intense of all of the transitions, and in some cases can be very difficult to distinguish from $Q_x$ vibronic overtones. (B)Chl’s are divided into three subgroups characterized by the degree of unsaturation of the macrocycle: porphyrin-type (fully unsaturated macrocycle), chlorin-type (single bond between C-17 and C-18 carbons in the macrocycle), and bacteriochlorin-like (single bonds between C-7 and C-8 as well as C-17 and C-18 carbons in the macrocycle).

The three representatives of the chlorin-like bacteriochlorophyll (BChl) subfamily called BChl c, d, and e (Figure 1) are employed by green sulfur photosynthetic bacteria (Chlorobiales) as primary photosynthetic pigments and assembled into large antenna complexes called chlorosomes. An individual chlorosome is usually composed of 100 000 to 250 000 self-assembled but non-covalently bound BChl molecules enclosed in a lipid monolayer envelope. The chlorosome also contains carotenoids that enhance spectral coverage and quench BChl triplet states, quinones to control environmental redox state and CsmA baseplate protein, as well as other proteins all

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Intensity Dependence of the Excited State Lifetimes and Triplet Conversion Yield in the Fenna–Matthews–Olson Antenna Protein

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Supporting Information

ABSTRACT: The Fenna–Matthews–Olson (FMO) protein is a soluble light-harvesting, bacteriochlorophyll a (BChl a) containing antenna complex found in green sulfur bacteria. We have measured time-resolved fluorescence and transient absorption at variable laser intensities at 298 and 77 K using FMO protein from Chlorobaculum tepidum prepared in both oxidizing and reducing environments. Fitting of the spectroscopic data shows that high laser intensities (i.e., above $10^{15}$ photons $\times$ cm$^{-2}$ delivered per laser pulse) distort the intrinsic decay processes in this complex. At high laser intensities, both oxidized and reduced FMO samples behave similarly, exhibiting high levels of singlet–singlet annihilation. At lower laser intensities, the reduced protein mainly displays a singlet excited state lifetime of 2 ns, although upon oxidation, a 60 ps lifetime dominates. We also demonstrate that the apparent quantum yield of singlet–triplet intersystem crossing in the reduced FMO complex is $\sim$11% in the most favorable low laser intensities, with this yield decreasing and the probability of singlet–singlet annihilation yield increasing as laser intensity increases. After correcting for stimulated emission effects in the experiments, the actual maximum triplet yield is calculated to be $\sim$27%. Experiments at 77 K demonstrate that BChl a triplet states in FMO are localized on pigments no. 4 or 3, the lowest energy pigments in the complex. This study allows for a discussion of how BChl triplets form and evolve on the picosecond-to-nanosecond time scale, as well as whether triplet conversion is a physiologically relevant process.

INTRODUCTION

Photosynthesis is the metabolic process by which solar energy is transduced into chemical energy.¹² Large pigment-containing antenna complexes absorb solar energy and transfer it to reaction center complexes that use the energy to transfer electrons. Although the reaction centers can be divided into two distinct, conserved groups, the antenna complexes absorb solar energy and transfer it to reaction center complexes that use the energy to transfer electrons. Although the reaction centers can be divided into two distinct, conserved groups, the antenna complexes are diverse across taxa and are uniquely tuned to each environment's light niche.³⁴ The anaerobic green sulfur bacteria Chlorobi and the recently discovered phototrophic acidobacterium Chloracidobacterium thermophylum contain two light-harvesting complexes: the chlorosome and the Fenna–Matthews–Olson (FMO) protein.³⁵–⁷ The chlorosome transfers energy to the FMO complex, which in turn transfers energy to the reaction center. Together, the chlorosome and FMO protein act as a funnel and wire, respectively, to aid in light absorption by the reaction center.⁸–¹⁰

Within the FMO complex itself, energy transfer between individual pigments cannot be explained by semiclassical energy “hopping” mechanisms alone; there is compelling evidence that wave-like quantum coherence occurs on the femtosecond-to-picosecond time scale.¹¹–¹⁴ The close intermolecular distances between pigments inside the complex lead to exciton coupling in which the chromophores can be described as in a superposition of several states. These exciplex transitions and energies have been investigated both experimentally and computationally.¹⁵–¹⁸

Structurally, the FMO protein complex is a homotrimer containing seven BChl a pigments bound inside each monomer with an eighth BChl a bound in a cleft between monomers, totaling 24 BChl a pigments in a fully intact complex.¹⁹–²² The FMO protein is unlike every other known light-harvesting complex in that it lacks association with carotenoids, which aid in light absorption and (B)Chl triplet state quenching. The FMO protein should therefore be subject to increased oxidative damage in the presence of molecular oxygen. To seemingly combat this, the FMO protein displays redox-dependent energy transfer behavior: in an anaerobic environment, the protein’s fluorescence lifetimes are on the order of $\sim$2 ns, but in aerobic environments, this lifetime shrinks to $\sim$60 ps, indicating the presence of a redox-dependent excitation quenching pathway whose molecular mechanism is unknown.²³–²⁶

For at least 30 years, time-resolved pump–probe spectroscopic experiments, such as transient absorption (TA), have been performed on this complex at room and cryogenic temperatures to probe behaviors of its pigments and to isolate individual pigments or groups of pigments for analysis (Figure 1).¹⁵

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Evidence of functional trimeric chlorophyll a/c2-peridinin proteins in the dinoflagellate Symbiodinium

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A B S T R A C T

The chlorophyll a-chlorophyll c2-peridinin-protein (apcPC), a major light harvesting component in peridinin-containing dinoflagellates, is an integral membrane protein complex. We isolated functional acpPC from the dinoflagellate Symbiodinium. Both SDS-PAGE and electrospray ionization mass spectrometry (ESI-MS) analysis quantified the denatured subunit polypeptide molecular weight (MW) as 18 kDa. Size-exclusion chromatography (SEC) and blue native gel electrophoresis (BN-PAGE) were employed to estimate the size of native acpPC complex to be 64–66 kDa. We also performed native ESI-MS, which can volatilize and ionize active biological samples in their native states. Our result demonstrated that the native acpPC complex carried 14 to 16 positive charges, and the MW of acpPC with all the associated pigments was found to be 66.5 kDa. Based on these data and the pigment stoichiometry, we propose that the functional light harvesting state of acpPC is a trimer. Our bioinformatic analysis indicated that Symbiodinium acpPC shares high similarity to diatom fucoxanthin Chl a/c binding protein (FCP), which tends to form a trimer. Additionally, acpPC protein sequence variation was confirmed by de novo protein sequencing. Its sequence heterogeneity is also discussed in the context of Symbiodinium eco-physiological adaptations.

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1. Introduction

Dinoflagellates are ubiquitous alveolate protists, closely related to apicomplexans and ciliates [1–3]. They have diverse morphology, genetics and different trophic modes (mixotrophic, heterotrophic and phototrophic). Photosynthetic dinoflagellates substantially contribute to the net primary production on Earth. Most of them are free-living species, while eight genera contain symbiotic representatives. Among them, Symbiodinium is the most commonly found genus of dinoflagellates in symbiosis with marine invertebrates and protists e.g. Cnidaria, which includes coral reef builders [4]. Symbiodinium provides corals their coloration and a variety of photosynthetically-produced nutrients; in return, corals supply the endosymbionts carbon dioxide, nitrate, phosphate and other inorganic substances that are essential for photosynthesis. Under certain conditions, when the photosynthesis of Symbiodinium is affected or impaired, and the endosymbiotic relationship cannot be maintained, corals start to expel Symbiodinium, leading to coral bleaching, which can significantly decrease the diversity of marine ecosystems. Coral bleaching can be caused by a number of biotic and abiotic factors, among which increased temperatures and solar irradiance are the most extensively studied. Under thermal stress, the Photosystem II (PSII) of Symbiodinium is inhibited to protect the cell from reactive oxygen species generated by excess electrons, which originate from PSII charge separation [5–8]. During this process, energy donors to PSII, namely Light Harvesting Complexes (LHCs), are thought to partially disconnect from PSII to reduce the level of excitations funneled to it [5]. The cellular level of LHCs also drops [7], preventing further damage. Although Symbiodinium LHCs are of considerable significance, the molecular level understanding of these protein-pigment complexes is limited compared to that of their counterparts in higher plants, green algae, diatoms and photosynthetic bacteria.

There are two major LHCs in Symbiodinium: the water-soluble peridinin-chlorophyll a-proteins (PCPs) and the thylakoid intrinsic chlorophyll a-chlorophyll c2-peridinin-protein complex (apcPC). They both contain peridinins, the unique carotenoid to dinoflagellates, as a major photosynthetic pigment. PCP has no sequence similarity to other LHCs [9]. Because of its uniqueness, PCP has been the subject of intensive experimental and theoretical studies [10–23]. In general, dinoflagellate PCP proteins are varied in the aspects of the length, pigment content, sequence and spectroscopic
Chemical activation of the cyanobacterial orange carotenoid protein

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The effects of the Hofmeister series of ions on the activation of the orange carotenoid protein (OCP) from the inactive orange form to the active red form were tested. Kosmotropes led to lower OCP activation, whereas chaotropes led to greater OCP activation. Concentrations of thiocyanate exceeding 1.5 M dark activate the orange carotenoid protein to its red form. This chemically activated OCP was studied by UV–vis and circular dichroism spectroscopies. The chemically-activated OCP quenches the fluorescence of phycobilisomes in vitro, to a level comparable to that of the light-activated OCP.

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1. Introduction

Light availability to photosynthetic organisms changes rapidly throughout the day due to changes in the sun’s position, cloud movement, sunflecks, and sun patches [1]. Intermittent periods of high light can over-saturate photosynthetic capacity. If left unregulated, excess absorbed photons can produce reactive oxygen species, resulting in cell damage or death [2,3]. Photosynthetic organisms have evolved multiple strategies to prevent oxidative damage from light stress, including decreasing antenna size and non-photochemical quenching [4]. NPQ provides a rapid valve, on a timescale of seconds to minutes, to dissipate excess absorbed light. The mechanisms of NPQ have been described for cyanobacteria [9,10], algae, and plants. Members of the light-harvesting complex (LHC) superfamily initiate NPQ upon sensing low luminal pH in plants and algae [5,6]. Cyanobacteria, however, sense rapid exposure to high light using a blue-light photosensor, called the orange carotenoid protein (OCP) [7,8].

The orange carotenoid protein is a 35 kDa protein involved in photoprotection in many cyanobacteria [9,10]. It functions as a sensor of light intensity, using the carotenoid 3'-hydroxyechinenone as a chromophore [11,12]. Upon exposure to extended periods (minutes) of high light, inactive, orange OCP (OCP0) becomes the active, red form (OCP1) [12], and rapidly quenches the phycobilisome antenna complex in vitro [13]. When the OCP gene is disrupted in Synechocystis sp. PCC 6803, no quenching of the phycobilisome is observed [7]. Overexpression of OCP leads to increased fluorescence quenching in Synechocystis [12]. Overexpressed OCP primarily binds echinenone due to limiting amounts 3'-hydroxyechinenone in the cell [14]. Echinenone-OCP is able to photoconvert and function similar to 3'-hydroxyechinenone—OCP. Deleting the crtO gene, required for the production of echinenone and 3’-hydroxyechinenone, results in OCP binding zeaxanthin [14]. Zeaxanthin-OCP appears yellow and is not photoactive [14]. As expected, the ΔcrtO strain lacking phototactive OCP fails to quench the phycobilisome [14]. These experiments have been confirmed by in vitro reconstitution of OCP with purified phycobilisomes in which only the red form is competent to quench [13]. The photoconversion properties of OCP are critical to its function as a photoprotective protein.

The conformational changes associated with OCP activation are significant [15]. OCP has two domains held together by a linker region. In the crystal structure, the N-terminal domain consists of two four-helix bundles, whereas the C-terminal domain is a member of the nuclear transport factor 2 superfamily [16]. Structural changes occurring between the N- and C-terminal domain occur upon activation. The active form appears to have a more open conformation and greater solvent exposure to 3’-hydroxyechinenone. Light-induced Fourier transform infrared (FTIR) difference spectroscopy showed that α-helices are less rigid and the β-sheets are more compact in the active form [12]. Time-dependent mass spectroscopy-based carboxyl footprinting of OCP in the red and

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