

REDOX REGULATION OF PHOTOSYNTHESIS BY THE CYTOCHROME *bf* COMPLEX: MECHANISMS AND CONSEQUENCES

By Paul J. Brantmier

Photosynthesis is a fundamental and central metabolic process of our planet. Honed by billions of years of evolution, it forms the basis for the food chain that sustains our ecosystem. Photosynthesis begins with sunlight capture by light harvesting complexes associated with photosynthetic membranes. The captured energy is funneled to photosystems II and I (PSII & PSI) reaction centers. In the PSII reaction center, light energy pulls electrons from water, evolving oxygen. These electrons drive a series of redox (oxidation-reduction) reactions passing through the cytochrome (Cyt) *bf* complex and PSI to generate chemical energy as adenosine triphosphate (ATP) and nicotinic adenine dinucleotide phosphate (NADPH). The danger in shuffling electrons through sequential redox transfers is the risk of unintended electron transfer to molecules such as oxygen, forming reactive radical species. The Cyt *bf* complex has been proposed as a source of superoxide, one of many forms of reactive oxygen species (ROS) that impair growth, function, and survival. My thesis addresses the regulation of electron transport and light harvesting processes by which photosynthetic organisms optimize light energy distribution between the photosystems (so called 'state transitions') and minimize ROS production. The Cyt *bf* complex has also long been implicated in sensing redox changes in electron transport and signaling the redistribution of light harvesting between PSII and PSI by mechanisms that are not well understood.

I used specific inhibitors and a sensitive fluorescent probe (H₂DCFDA) to characterize ROS production in the Cyt *bf* complex of the cyanobacteria (blue-green micro-algae), *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002. Inhibitors were also used in room-temperature and 77°K fluorescence studies to investigate the role of the Cyt *bf* complex in redox sensing and signaling that mediates the redistribution of light-harvesting phycobiliproteins (PBS) in these cyanobacteria. Findings from this work show that the Cyt *bf* complex plays a central role in these processes. These and previously published data allowed me to formulate a detailed model of redox signaling by the Cyt *bf* complex, and regulated redistribution of light energy by the formation of dynamic light-harvesting (PBS) supercomplexes involving the Cyt *bf* complex, PSII, and PSI. The model proposes two distinct means by which these 'state transitions' occur. I propose Cyt *bf* – independent and – dependent sensing-signaling mechanisms. The Cyt *bf* – dependent mechanism depends on the presence of light and appears to require binding events or conformational changes in the Cyt *bf* low-potential electron transfer chain or quinone-reductase (Q_n) site. I propose that the Cyt *bf* – independent mechanism lies downstream of the *bf* complex. Together, these signal the formation of PSII-PBS-Cyt *bf* and PSI-trimer-PBS complexes during illumination, and the predominant formation of PSI-monomer-PBS complexes during darkness. These in turn determine the relative light-harvesting capacities of PSII and PSI. The model and supporting evidence are discussed in the context of strategies that have evolved to maximize photosynthetic efficiency and minimize ROS production.

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COMPLEX: MECHANISMS AND CONSEQUENCES

by

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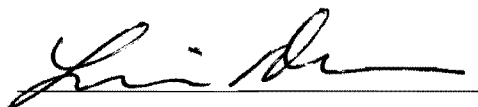
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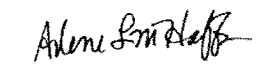
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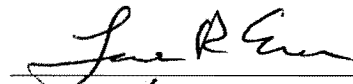
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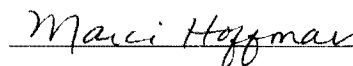
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Chapter 1: Introduction

1.1 – Reactive Oxygen Species and Electron Transport

1.1.1 – Reactive Oxygen Species (ROS).

Free radicals are unstable molecules that carry an unpaired electron and can react with, and thereby damage, cellular components and macromolecules. Many of the complexes of mitochondrial electron transport are encoded in the mitochondrial DNA (1). Oxidative damage to mitochondrial DNA has been shown to be ~10x higher than to nuclear DNA (2,3) suggesting that reactive oxygen species (ROS) could be a primary cause of this damage. Subsequent damage to electron transport-specific genes encoded in the mitochondrial DNA is thought to contribute to decreased mitochondrial electron transport efficiency by modifying the components of the electron transport chain increasing localized ROS production (1,4,5,6). This local production of ROS may further impair electron transport through reduction or other chemical modifications of integral components or lipids (lipid-peroxidation) the latter allowing protons to leak from one side to the other, inhibiting the establishment of a proton gradient. These conditions are thought to be the main contributors to age-related diseases and some forms of cancer (7). Primary producers of ROS in mitochondrial electron transport are NADH dehydrogenase (complex I) and the cytochrome *bc* complex (complex III).

Understanding how ROS are produced from these and other putative sources requires understanding the nature of oxidation/reduction reactions and how molecular oxygen is reduced to superoxide ($O_2^{\bullet-}$) and subsequently to other forms of ROS.

Chemically, electron transport consists of a series of coupled oxidation/reduction (redox) reactions. The mid-point potential of an oxidation/reduction couple (electron donor/acceptor pair) is a measure of the potential (in mV) required to reduce half of the substrate (the electron acceptor). Table 1 displays the mid-point potentials of several electron carriers in photosynthetic electron transport complexes and components. The oxidation/reduction (redox) couple, O_2 /superoxide, has a midpoint potential of -330 mV under standard conditions at 1 atm determined by a pulse radiolysis technique (8), which has been re-evaluated recently to -160 mV by quinone-electrode measurements, at physiological pH in solution (9). During the process of electron transport, electrons move down a series of energetically favorable redox reactions. The reactants are the molecular and protein components of the electron transport chain. Electrons move by themselves or together with a proton, between redox centers within protein complexes and extrinsic molecules such as quinones or cytochromes. Electrons pass through the electron transport chain components from lower (more negative mV values) to higher redox potentials (more positive mV values). The two electron reduction of O_2 to H_2O has a high redox midpoint potential and can thus occur readily and safely in the context of terminal oxidase enzymes. The one electron reduction of O_2 to superoxide ($O_2^{\bullet-}$) has a much lower midpoint potential and therefore occurs only in the vicinity of low potential electron donors. Because most electron transfer reactions take place within the membrane where oxygen is largely excluded, electron transfer to oxygen in this matter occurs infrequently, representing only 0.1-2% of electrons passing through the chain. (10) However, when a low potential electron transport component does accidentally transfer an electron to oxygen, superoxide is formed. Superoxide can be described as a highly-reactive, diatomic oxygen with a 'resonating' electron (10). Once superoxide damages

membrane phospholipids and/or membrane and redox components of the electron transport chain, the incidence of oxygen contacting the redox components can increase, creating even more superoxide and greater damage in a positive feed-back loop.

Table 1. – Redox Midpoint Potentials of Photosynthetic Electron Transport Components

Complex	Redox Component	Source	Species	Midpoint Potential	Ref.
Photosystem II					
	P680			(+) 1120 mV	100
	Pheophytin	Direct Titration	<i>Syn 6803</i>	(-) 640 mV	101
	Q_A	Direct Titration	<i>Syn 6803</i>	(-) 30 mV	101
	Q_B	Estimation		est. 0 mV	102
Plastoquinone Pool					
	$PQH_2/PQH\cdot (Q_0)$		<i>R. sphaeroides</i>	(+) 90 mV	103
	$PQH\cdot/PQ$ (semiquinone)			(-) 300 to (-) 400 mV	104
	$PQH\cdot/PQH_2 (Q_i)$		Yeast	(+) 110 mV	105
Cytochrome b_6f					
	b_h heme	Thylakoids	Spinach	(-) 45 mV	106
	b_p heme	Thylakoids	Spinach	(-) 150 mV	106
	c_n heme	Isolated Complex	<i>Chlamydomonas</i>	(+)100 mV	48
	Rieske Fe-S Cluster	Isolated Complex (pH<8)	Spinach	(+) 320 mV	107.108
	Cytochrome f	Isolated Complex	Spinach	(+) 340 mV	109
	Plastocyanin		<i>Chlamydomonas</i>	(+) 370 mV	110
Photosystem I					
	P700	Whole Cells	<i>Chlamydomonas</i>	(+) 447 mV	111
	A_1	Isolated Complex	Spinach	(-) 540 mV	112
	F_x		Cyanobacteria	(-) 670 mV	113
	F_A	Isolated Complex	Spinach	(-) 553 mV	114
	F_B	Isolated Complex	Spinach	(-) 594 mV	114
	Ferredoxin		Cyanobacteria	(-) 325 to (-) 390 mV	115

Table 1. – P680 and P700 indicate the special chlorophylls within photosystem II or I, respectively. All other redox components are explained in sections 1.2.1-1.2.4. The midpoint potentials were measured at pH 7 unless otherwise indicated. The midpoint potentials shown are for the oxidized/reduced couple of one electron carriers (e.g. $P680^+/P680$). For multiple electron carriers, the specific reaction is shown (e.g. $PQH_2/PQH\cdot$). Modified from Horn, D, 2004 (27).

In Table 1, note that the semiquinone generated by one electron oxidation of quinol (e.g. plastoquinol oxidation in the cytochrome b_6f complex) has a low midpoint potential and

thus could pass its unpaired electron to O_2 . Superoxide is a highly reactive radical and the precursor for several other forms of ROS and reactive nitrogen species (RNS) (2, 5).

Natural defenses and antioxidant systems have evolved to combat $O_2^{\bullet-}$ produced by electron transport. Superoxide dismutase is an enzyme that converts superoxide to hydrogen peroxide (Figure 1) at a rate limited only by diffusion (10). The conversion from $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2) requires either a pair of Mn or a Cu-Zn pairing of cofactors within the soluble superoxide dismutase protein to mediate the addition of two protons to a superoxide molecule. Cu-Zn-containing superoxide dismutases are generally found in eukaryotic cytosol whereas the Mn variety is found primarily in mitochondria. Zelko et al. (10) describes the fascinating dynamics of the enzymatic heritage suggesting an evolutionary progression from Fe-containing superoxide dismutase (prevalent in bacteria) toward Cu-Zn superoxide dismutase with Mn-containing superoxide dismutase as an intermediate form.

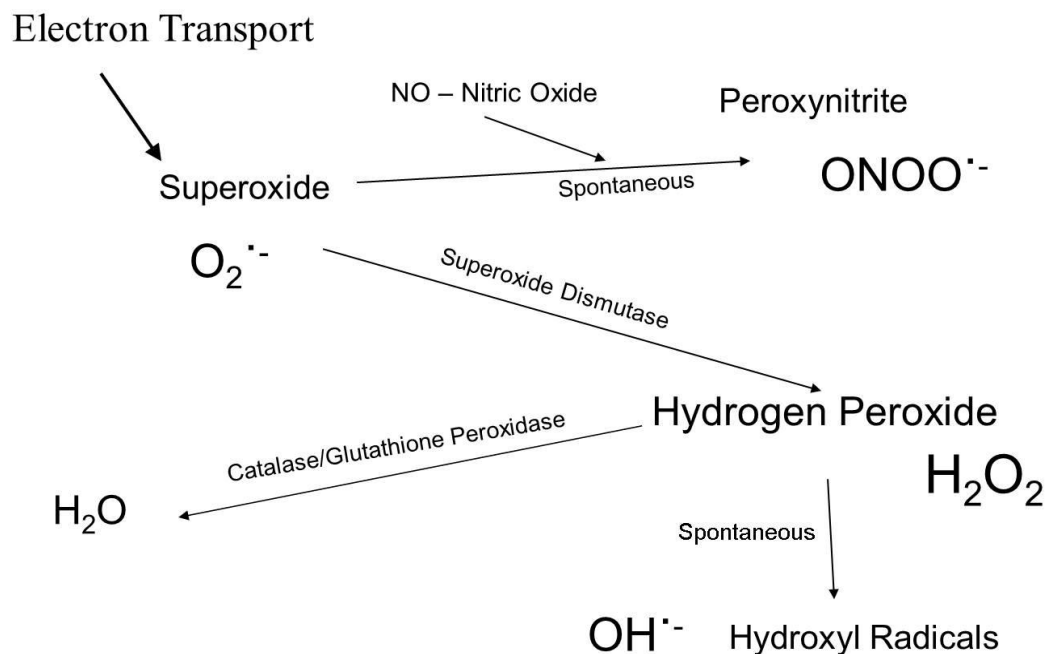


Figure. 1 – Reactive Oxygen Species Degradation/Mutation Pathways. Superoxide produced by electron transport can form peroxynitrite if contacting NO or form hydrogen peroxide through the activity of superoxide dismutase. Hydrogen peroxide can spontaneously degenerate into the extremely reactive hydroxyl radical ($\text{OH}^\bullet$) or be converted by the activity of catalase or glutathione peroxidase into water.

Catalase and glutathione peroxidase (Figure 1) are enzymes that form water from the less reactive ROS, hydrogen peroxide (11, 12). Generally found in the cytosol and sometimes excreted as a protective mechanism, catalase uses a heme cofactor to take two H_2O_2 molecules and form two H_2O molecules and an O_2 . Glutathione peroxidase is a sulfhydryl-containing enzyme requiring selenium as a cofactor to mediate cysteine-disulfide bond formation functions as a tetramer oxidizing two H_2O_2 molecules to four H_2O molecules leaving the sulfhydryl-containing cysteines as disulfide bonds. Glutathione peroxidase can be reset by glutathione reductase activity mediating NAD(P)H reduction of the cysteine disulfide bond. This series of enzymes takes superoxide and reduces it to water. Given that evolution has favored a system that

removes ROS one might hypothesize that other areas of electron transport have minimized ROS production while maximizing efficiency. One goal of this thesis is to determine whether rapid changes of the photosynthetic apparatus have evolved to minimize ROS production.

Additional ROS are noted in Figure 1. When superoxide combines with nitric oxide (NO) the peroxynitrite ($\text{ONOO}\cdot^-$) anion is formed. This form of ROS, and intermediate forms, are often implicated in direct damage to DNA. Hydrogen peroxide and superoxide apparently do not interact with nucleotides at all (2). Singlet oxygen is another form of ROS most prevalent in thylakoids during photosynthetic activity because it is created by excess excitation/resonance energy transfer from excited state chlorophylls to oxygen. This energy transfer changes the spin of the electrons in the outer-most shell to an antiparallel configuration making the oxygen molecule very reactive. However, singlet oxygen is not a byproduct of superoxide and for this reason is not shown in Figure 1. The hydroxyl radical ($\text{HO}\cdot$) is a degradation product of hydrogen peroxide (Figure 1). This radical is extremely reactive (12), reacting with nearly any molecule it encounters. Because hydrogen peroxide has a long half-life relative to other forms of ROS (12), one can think of it as a time bomb. The relatively inert hydrogen peroxide molecule diffuses through the cell until it degenerates spontaneously into hydroxyl radical, then causing damage to molecules in its vicinity. Lipid-peroxidation is another primary effect produced by most forms of ROS. Destruction of fatty acids by lipid peroxidation can make electron transport chains leaky to protons. Thereby more electrons passing through the electron transport chain are required to generate the proton gradient used to produce

ATP. This lowers the amount of ATP available for cellular processes. This inefficiency has been considered a primary cause of mitochondria isolated from ageing animals tending to be larger, have increased matrix vacuolization, shorter cristae, and a lower amount of dense granules (1).

1.1.2 – Electron Transport.

The process of transducing energy, in its various forms, through the membrane-bound protein complexes of electron transport has been studied extensively in mitochondria and thylakoids (for review 13, 14, 15, 16). In mitochondrial electron transport NADH (nicotine adenine dinucleotide) is oxidized by NADH dehydrogenase (NDH/Complex I) and in turn reduces intra-membrane ubiquinones to ubiquinol. The membrane-spanning cytochrome bc_1 complex (Complex III) oxidizes ubiquinol, subsequently reducing a soluble cytochrome c protein. Cytochrome Oxidase (COX/Complex IV) oxidizes cytochrome c and reduces O_2 to H_2O . Electron transfer through the thylakoids of cyanobacteria follows a similar scheme except one might expect, photosynthetic complexes photosystem II (PSII) and photosystem I (PS I) are the primary reducers and oxidizers, respectively, of the electron transport chain when light is present. Many higher plants and other chloroplast-containing organisms, such as the green alga *Chlamydomonas reinhardtii*, have both mitochondrial and photosynthetic electron transport chains, compartmentally separated. In these plant and algal chloroplasts, electron transport is primarily catalyzed by the photosystem reaction centers whereas respiratory electron transport occurs in the mitochondria. In cyanobacteria, the two electron transport chains are joined via several components. The thylakoids of

cyanobacteria accommodate NADH dehydrogenase (NDH) and succinate dehydrogenase (SDH), in addition to PS I and PS II complexes, which also reduce plastoquinone to plastoquinol to be oxidized by the cytochrome *bf* complex. Oxidation of the electron transport chain in cyanobacteria can be accomplished by PS I or cytochrome oxidase (COX). The cytochrome *bc* complex of mitochondrial electron transport is structurally and functionally related to the cytochrome *bf* complex in cyanobacteria and chloroplasts.

Thus in electron transport chains, electrons flow from primary, reductant-generating complexes (NADH dehydrogenase/PSII) to intra-membrane lipophilic quinones (Ubiquinone/Plastoquinone) to intermediate proton-pumping cytochrome reductases (cytochrome *bc/bf* complexes), that reduce soluble electron carriers (cytochrome *c*/plastocyanin), that are oxidized by terminal oxidases in respiratory (mitochondrial) electron transport or PS I in photosynthetic electron transport. Thus, mitochondrial and photosynthetic electron transport follow similar schemes (Complex I > Complex III > Complex IV in mitochondria and PS II > Complex III > PS I in photosynthesis). Exceptions, details, and elaborations of these general schemes are described below in section 1.1.3. These comparisons are important for understanding the compensatory dynamics of electron flux, specifically the mechanism by which organisms, using electron transport, sense the oxidation/reduction (redox) potential of electron carrier components within their electron transport chains. Numerous genes and proteins have been identified whose expression levels change-in response to redox modulation of electron transport (17) However, the understanding of redox activation and signaling mechanisms has remained elusive although interesting findings are slowly

emerging on several fronts (see 18, 19). This thesis seeks to probe the relationship between redox-regulation of photosynthetic electron transport and the production of reactive oxygen species.

1.1.3 – Reactive Oxygen Species and Electron Transport – Consequences and Implications.

Generally, it has been found that photosynthetic organisms are better than non-photosynthetic organisms at regulating the production of reactive oxygen species (19). Thus, photosynthetic organisms are subject to less damage by ROS to their electron transport systems, likely because of more potent antioxidant defense mechanisms and signaling cascades that mobilize stress responses. Understanding how photosynthetic organisms have learned to cope with high levels of reactive oxygen species may open avenues of treatment for mitochondrial degenerative diseases and disorders. One example is that plants tend to have very high levels of ascorbate, a ROS scavenger. Treatments with ascorbate in humans have had some benefits although they have not been effective in preventing aging and cancer (2). Plants and photosynthetic microorganisms clearly have many other mechanisms to help shield their macromolecules from ROS damage and this provides one practical rationale for investigating such mechanisms.

Eukaryotic photosynthetic organisms have both mitochondria and chloroplasts. The primary contributors to ROS production in these organisms are the chloroplasts themselves, producing some 18 times the amount of ROS as their mitochondria (19). These values were estimated from respiratory and photosynthetic rates combined with the rates of oxygen reduction to superoxide. This extraordinary overproduction of ROS in

chloroplast is thought to be due to the low redox potentials of intermediates, such as primary electron acceptors in PS I (-0.9 mV), and their proximity to the substrate (O_2) produced by PSII. Currently, most methods for quantification of ROS are less discriminatory than needed to determine specific sources and types of ROS. Many probes used for quantification interact equally well or to some degree with superoxide, singlet oxygen, and other forms of ROS such as hydroxyl radicals and hydrogen peroxide. This may lead to over-estimation of the damage caused by ROS increases. For example, in photosynthesis, accelerated photosystem II reaction center, D1 protein turnover is quite rapid, likely because singlet oxygen is produced in copious amounts at or near the PS II reaction center. However, singlet oxygen and superoxide are thought to have very short half-lives (1ns-1 μ s) and thus the damage is localized (20). Hydrogen peroxide, however, has a lengthy half-life in solution (8 hours to 20 days) suggesting that H_2O_2 accumulation could be far more destructive (21, 22). It is under these conditions that discrimination of the various forms of ROS becomes important for estimating and understanding the impacts of ROS. Understanding cellular detoxification capacities also becomes important. Plants contain large amounts of known redox buffering compounds ascorbate (10-100 mM) and glutathione (0.2–10 mM) used by ascorbate and glutathione peroxidases, respectively (see section 1.1.2). These compounds effectively regulate the level of H_2O_2 and its detrimental impacts. Furthermore it is suggested that changes in H_2O_2 concentrations may act as a form of signaling between chloroplast, mitochondrial, and peroxisomal compartments. Within the chloroplast, ascorbate, and a

ferredoxin/thioredoxin system have been found to serve as redox-sensitive signaling components (19).

In mitochondria, hydrogen peroxide acts as a signaling molecule that initiates the mitochondrial permeability transition pore involved in signaling apoptosis (7). The subsequent release of cytochrome *c* from mitochondria signals a caspase-mediated cascade leading to apoptosis (7, 5). In plants, chloroplasts have taken over this task of initiating programmed cell death (23). During leaf senescence in spinach the expression of NDH (NADH dehydrogenase) increases in chloroplasts without an accompanying increases in SOD (superoxide dismutase). The resulting rise in ROS induces programmed cell death via cytochrome *c* release from the plant mitochondria (described immediately above). Thus the H₂O₂-specific mechanism of regulating programmed cell death (mitochondrial release of cytochrome *c*) seems to be conserved between the plant and animal kingdoms, although adapted to different, primary energy transducing, electron-transport chains. This and other evidence implicates H₂O₂ as a fundamentally important signaling molecule in higher plants. It is thought that damage from H₂O₂ is largely limited by the H₂O₂ signaling mechanism as H₂O₂ production is compartmentalized both in chloroplasts and mitochondria. Cyanobacteria have thylakoid membranes but do not show the granal and stromal membrane regionalization of their photosystems as in eukaryotes. Thus the oxygen substrate for superoxide is produced by photosystem II in even closer proximity to powerful sources of reduction in PS I and the cytochrome *bf* complex. Superoxide production and subsequent conversion to hydrogen peroxide have dramatic, immediate impacts on photosynthesis, because concentrations as low as 10 μ M

H₂O₂ have been shown to impede CO₂ fixation by 50% in *Synechococcus* Strain PCC 7942 (21, 22).

A microarray study recently analyzed the impact of hydrogen peroxide (17) on *Synechocystis* PCC 6803. A previous genomic survey had revealed an open reading frame (slr1738) similar to the PerR peroxide responsive transcription regulator of *Synechocystis* PCC 6803 (24). Slr1738 knockout mutants were compared against the wild type after exposure to hydrogen peroxide (1.5 mM to 75 μ M) for 15 minutes. Numerous genes were stimulated in response to hydrogen peroxide although the study focused on those that were slr1738-dependent. Genes down-regulated in the Δ PerR mutant compared to the wild type included: Unknown/Hypothetical proteins, peroxiredoxins, and Fur-type Transcription factors. This study shows that cyanobacteria possess specific proteins for sensing hydrogen peroxide and subsequent transcriptional regulation. It is not yet clear whether cyanobacteria produce H₂O₂ specifically as a second messenger (24, 25).

In cyanobacterial photosynthetic electron transport, PS II and PS I provide the primary means of reducing the electron transport chain in the presence of light. Complexes I (NADH/NADPH dehydrogenase or NDH) and II (succinate dehydrogenase or SDH) are the primary reducers of the electron transport chain in the dark (26). In cyanobacterial electron transport, the cytochrome *bf* complex has been shown to contribute significantly to the ROS burden of the cell (27). In particular, mutations that alter electron flux within the *bf* complex can significantly increase ROS production in *Synechococcus* PCC 7002, resulting in slowed growth and perturbation of the redox balance and distribution of light-harvesting proteins (27, Ch. 3) Thus, throughout the

domains of life, ROS is a burden to organisms using electron transport to produce energy, contributing to ageing and cancer in higher eukaryotes and slowing growth and affecting energy redistribution in cyanobacteria. The almost universal existence of ROS defense mechanisms such as superoxide dismutase and catalase (together reducing dangerous superoxide to harmless water) further indicates that ROS are a significant and widespread threat to growth, function, and survival. Thus identifying the sources and mechanisms of ROS production by the cytochrome *bf* complex of photosynthesis and their subsequent consequences on basic cellular processes has broad relevance for understanding how organisms of all kinds have learned to cope with highly toxic oxygen species.

1.2 - Structure and Function of Electron Transport Chains

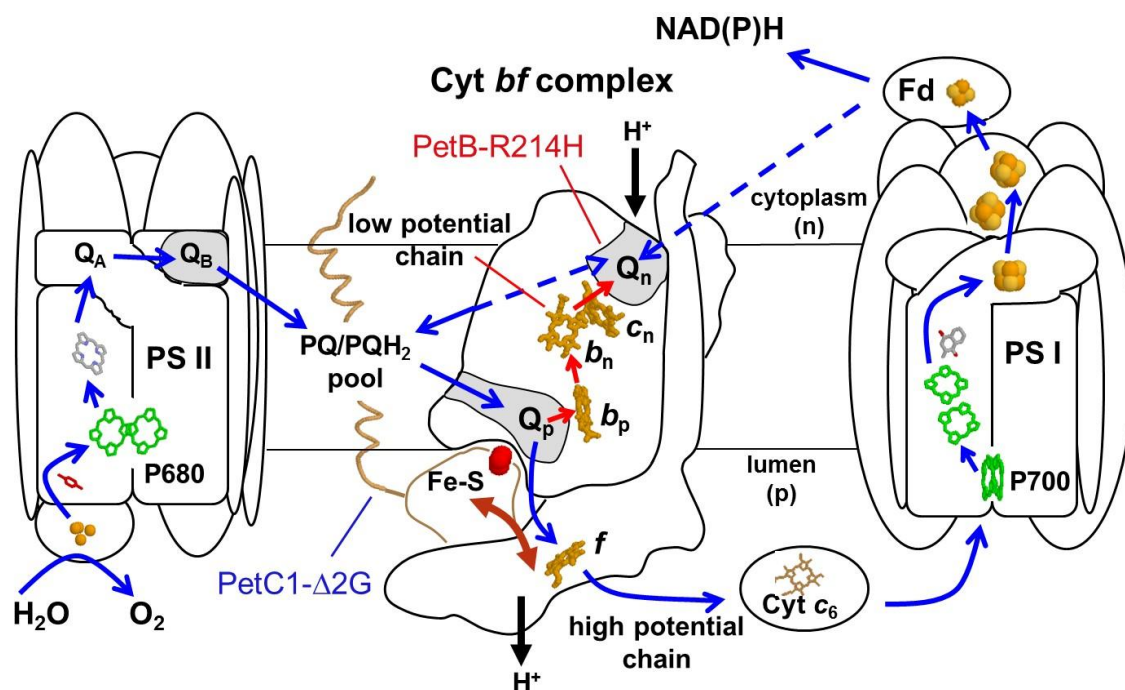


Figure. 2 – The Photosynthetic Electron Transport Chain. PSII absorbs light energy (via P680 chlorophyll) stripping electrons from water on the lumenal side of the thylakoid membrane. Electrons are then transferred to intrinsic quinones bound inside PSII Q_A and Q_B . PSII reduces plastoquinone (PQ) to plastoquinol (PQH₂). PQH₂ are oxidized at the quinol oxidation (Q_p) site of the cytochrome *bf* complex (Cyt *bf*) and cytochrome *c*/ plastocyanin (Cyt *c*/PC) are reduced and then oxidized by light energy captures by PSI. The resulting reduced ferredoxin (Fd) can then reduce NADPH via ferredoxin NADPH reductase or it is thought might participate in cyclic electron flow via reduction of quinones at the Cyt *bf* quinone reductase (Q_n) site. Diagram courtesy of Darryl Horn, 2004.

1.2.1 – Photosynthesis.

Photosynthetic organisms harvest light through the use of molecular antennae and funnel the energy to photosystem reaction centers (for reviews see 14, 15, 28). Within these reaction centers the light energy is transduced resulting in the generation of electrons and subsequent redox reactions to move protons across the membrane as illustrated in Figure 2 and explained in greater detail below. This process of electron transport establishes the proton motive force used to drive adenosine triphosphate (ATP) synthesis via ATP synthase. The ultimate electron acceptor of the photosynthetic electron

transport chain is CO_2 , which is reduced with electrons from ferredoxin via NADPH, ribulose 1,5-bis-phosphate carboxylase/oxygenase (Rubisco) and the Calvin-Benson Cycle resulting in carbohydrate synthesis. Photosynthesis produces carbon polymers, O_2 , and many ATP-dependent biosynthetic products. These in turn provide energy for the metabolic processes of non-photosynthetic organisms.

The “purpose” of photosynthetic electron transport is to translocate protons across the thylakoid membrane producing the proton motive force (pmf) that generates ATP and reducing power in the form of ferredoxin and NADPH as described below in greater detail (16, 29). The pmf is comprised of a chemical (ΔpH) and electrical component ($\Delta\Psi$). The translocation of protons from the stromal or cytoplasmic side of the membrane (negative or N-side) to the luminal side (positive or P-side) produces both a chemical and electrical gradient used to produce ATP. The photosynthetic electron transport chain is a series of membrane-bound complexes Photosystem II (PSII), the cytochrome *bf* complex, and Photosystem I (PS I). The photosystems (PS I and PSII) are reaction centers that convert light energy into electrical/redox energy. This electrical energy produced at the reaction centers then “flows” in the form of electrons from more negative redox coupling midpoint potentials to more positive ones (Table1). The linear electron transport chain begins with harvested light energy funneled to PSII. The resonance energy funneled to the reaction center via the light harvesting antennae excites chlorophylls within PSII. Electrons from the excited-state reaction center chlorophylls enter the electron transfer chain by reducing pheophytin and other electron carriers within the PS II reaction center complex. The resulting strong oxidant generated by the oxidized PS II reaction center

chlorophyll extracts electrons from water. Electrons from water flow via the manganese cluster and tyrosine residues to re-reduce the oxidized PS II reaction center chlorophyll molecules. The funneled resonance energy from the light-harvesting antennae can then excite these reaction center chlorophylls to start the photochemical process again. The manganese cluster is located on the positive (P) side of the membrane. The subsequent splitting of H_2O into O_2 and 2H^+ contributes to the proton gradient. Reduced pheophytin within PS II in turn reduces quinone_A (Q_A), a quinone bound within PS II near the N-side of the membrane, in turn reducing quinone_B (Q_B).

Quinone_B is a plastoquinone molecule that is not bound tightly to the PS II complex as is quinone_A. When reduced with two electrons by two turnovers of the PS II complex, the lipophilic plastoquinone picks up two protons from the stromal or negative (N) side of the membrane. The reduced plastoquinone (plastoquinol or PQH_2) diffuses within the membrane to the quinol oxidation site of the cytochrome *bf* complex on the P-side of the membrane. Here the oxidation of plastoquinol (PQH_2) deposits the two protons from the stromal (N) side onto the luminal, P-side of the membrane. In this oxidation, the first electron from plastoquinol passes through the cytochrome *bf* high potential chain through the hinged Rieske Iron sulfur protein to cytochrome *f*. The second electron from plastoquinol oxidation passes into the cytochrome *bf* low potential chain as described further below.

The soluble electron carriers cytochrome *c*₆ and/or plastocyanin accept the electron from cytochrome *f* on the luminal (P) side of the membrane and dock with PS I. When the PS I P700 chlorophyll reaction center is excited with harvested light energy,

the P700 reaction center chlorophyll becomes oxidized and the electron travels through a series of iron-sulfur clusters to the stromal (N) side, soluble electron carrier ferredoxin. The oxidized PS I reaction center is re-reduced with an electron from the lumen-side soluble carrier cytochrome c_6 or plastocyanin. Within PS I, this electron passes through a series of iron-sulfur clusters ('F' denoting an iron-sulfur cluster and subscripts indicating a distinct iron-sulfur cluster) $F_A > F_B > F_X$ to the stromal electron carrier ferredoxin as shown in Figure 2. Ferredoxin in turn can reduce NADP⁺ (via ferredoxin-NADP reductase) to form NADPH which provides reductant for numerous metabolic processes. Alternatively, electrons from ferredoxin can enter a cyclic pathway to reduce plastoquinone either directly via the cytochrome *bf* quinone reductase site as described below (section 1.2.4) or via NADPH and NADPH dehydrogenase. These cyclic pathways result in ATP synthesis at the expense of NADPH formation. Thus, the cytochrome *bf* complex plays essential roles in electron flow for NADP reduction, generating the proton motive force for ATP synthesis, and mediates linear versus cyclic electron for determining the cellular ATP/NADPH ratio.

1.2.2 – Respiration: Protein Complexes and Functions.

Respiratory electron transport (Figure 3) chains exist in some form in all photosynthetic organisms. Photosynthetic eukaryotes have mitochondria in addition to chloroplasts and thus a full complement of mitochondrial and photosynthetic electron transport components (14, 26). Cyanobacteria, such as *Synechocystis* PCC 6803 (*Synechocystis*) and *Synechococcus* PCC 7002 (*Synechococcus*), have respiratory components interwoven with the photosynthetic complexes of the thylakoid membrane

and subsequently use plastoquinone as the lipophilic quinone exclusively. Henceforth I refer to *Synechocystis* unless otherwise noted. It is suggested that the NADH dehydrogenase complex in these cyanobacteria may function more like an NADPH dehydrogenase because it preferentially oxidizes NADPH (26). Regardless, succinate dehydrogenase seems to be the primary reducer of the plastoquinone pool during respiratory electron transport, at least in *Synechocystis* (26).

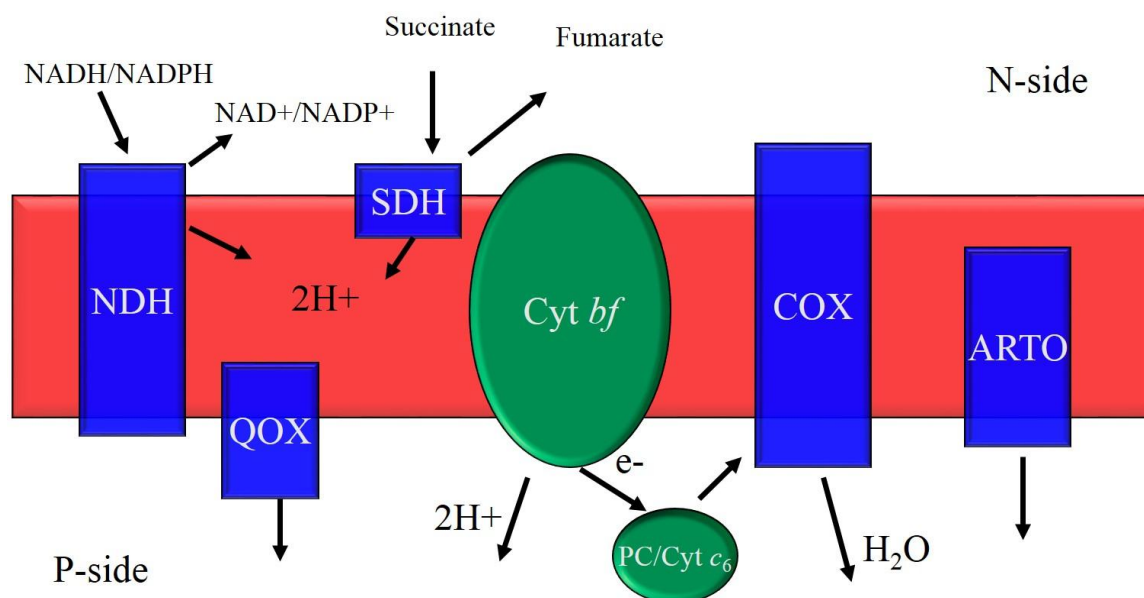


Figure 3. The Respiratory Electron Transport Chain of Unicellular Cyanobacteria. The components shown apply to *Synechocystis* PCC 6803. The plastoquinone (PQ) pool within the thylakoid membrane is shown in red. NADH/NADPH dehydrogenase (NDH) pulls reductive potential from NADH or NADPH. Succinate Dehydrogenase (SDH) pulls reductive power from succinate. Quinol Oxidase (QOX) oxidizes plastoquinol (PQH_2) to plastoquinone (PQ). Cytochrome Oxidase (COX) oxidizes reduced plastocyanin/cytochrome *c* (PC/Cyt *c*₆) and reduces oxygen to water. The cytochrome *bf* complex (Cyt *bf*) oxidizes quinol to quinone and reduces PC/Cyt *c*₆. Alternative Respiratory Terminal Oxidases (ARTO) also oxidize the PQ pool.

NADH/NADPH and succinate are the primary substrates through which the respiratory chain reduces plastoquinone to PQH_2 . In *Synechocystis* two NADH dehydrogenase complexes exist. NDH-1 and NDH-2 could instead be considered an

NADPH dehydrogenase and an NADH dehydrogenase, respectively, indicating their substrate preferences. NDH-2 is a much smaller complex and encoded by an operon with only three genes. NDH-1, in size, more closely resembles the multi-protein mitochondrial complex I yet prefers NADPH to NADH as evolution might dictate considering the high ratio of NADPH:NADH in cyanobacteria (26). Mitochondrial complex I produces copious amount of ROS (reactive oxygen species, defined above - section 1.1.1) when down-stream oxidation of the ubiquinone pool is blocked at the cytochrome *bc* complex or in the presence of rotenone, inhibiting reduction of the ubiquinone pool via complex I (30). Thus NDH-1 might be a similar source of ROS in photoheterotrophic cyanobacteria.

Succinate dehydrogenase oxidizes succinate and uses the energy to reduce a bound flavin molecule FAD (flavin adenine dinucleotide) to FADH₂. The complex then uses the reduced FADH₂ to reduce a quinone to quinol. Succinate dehydrogenase has not been implicated as a source of ROS production despite the presence of low midpoint-potential components (Table 1), because the reaction site is not accessible to O₂. This complex is reportedly (26) inhibited by malonic acid; however, I have not found experimental evidence to support this conclusion in *Synechocystis*. HQNO (2-n-heptyl-4-hydroxyquinoline-N-oxide), a cytochrome *bf* quinone-reductase site inhibitor, has been used to determine the quinone binding affinity of SDH in *Escherichia coli* (31) suggesting that HQNO and its analog NQNO (2-n-nonyl-4-hydroxyquinoline-Noxide) might inhibit the succinate dehydrogenate complex as well.

Thus the primary protein complexes capable of reducing the plastoquinone pool in cyanobacterial respiration are NADH/NADPH dehydrogenases and succinate

dehydrogenase. The plastoquinone pool is oxidized by means of the cytochrome *bf* complex, *bd*-type quinol oxidases (encoded by *cyd* genes), and/or Alternative Respiratory Oxidases (ARTO). Quinol oxidases/peroxidases use the reductive potential of quinols directly to reduce O_2 to H_2O (oxidases) or H_2O_2 (peroxidases) (13). *Synechococcus* PCC 7002 apparently does not contain quinol oxidases of any type (32). As in other cyanobacteria, the cytochrome *bf* complex of *Synechococcus* reduces cytochrome c_6 which is the soluble electron carrier that can dock with cytochrome oxidases (Complex IV), either the typical *aa*₃-type terminal oxidases (CtaI) and apparently with the alternative respiratory terminal oxidases (CtaII) as well. These heme-copper oxidases can use the reductive potential from cytochrome c_6 to reduce O_2 to H_2O (13). NQNO, HQNO, pentachlorophenol, and KCN are known to inhibit quinol oxidases whereas the most efficient inhibitor of cytochrome oxidase seems to be KCN (33).

1.2.3 - Structure of the Cytochrome *bf* Complex

The cytochrome *bf* complex (Cyt *bf*) is a membrane-bound protein complex consisting of four major subunits; the Rieske iron-sulfur protein (PetC), cytochrome b_6 (PetB), subunit IV (PetD), and cytochrome *f* (PetA) (34, 35, 36, 28, Figure 4). This complex is essential for the quinone-mediated proton translocation in linear photosynthetic and respiratory electron transport and in photosynthetic, cyclic electron transport. Cytochrome *bf* complexes are related in terms of overall structure and function, quinone-binding sites, and most of the prosthetic groups to cytochrome *bc* complexes of bacteria and mitochondria including *Homo sapiens*. Cytochrome *bf*

complexes also function in plastoquinone-pool, redox-dependent signaling of photosynthetic regulatory responses.

The core structure of the cytochrome *bf* complex is defined by seven membrane spanning α -helices (36, 35) encoded by the *petBD* dicistronic operon in cyanobacteria (37). The cytochrome *b₆* polypeptide forms a series of four membrane-spanning helices that form a vertical cylinder and provides ligands for the electron-conducting hemes of the low potential chain (35, 36). The *petD* product, subunit IV (SUIV), provides support for the chlorophyll *a* molecule clamping the ‘head’ of the molecule between two transmembrane helices while the tail is supported between the SUIV head-clamping helices and two cytochrome *b₆* core membrane spanning helices. Additionally the SUIV protein’s transmembrane helices help form the quinol-oxidation (Q_p) site (35). The 23 kDa cytochrome *b₆* and 17 kDa subunit IV of the cytochrome *bf* complex are homologous to 42 kDa cytochrome *b* (38) of the bacterial-mitochondrial cytochrome *bc₁* complex. The *petC* gene encodes the Rieske Iron-Sulfur Protein (RISP). This structure includes a hydrophobic α -helical, membrane-spanning tail that anchors the protein to the membrane. The N-terminus protrudes from the stromal side of the membrane. The carboxy-terminal 2Fe-2S head region of the protein is attached to the luminal side of the membrane anchor via a flexible, glycine hinge. This iron-sulfur cluster domain swings between a proximal position at the quinol oxidation site and a distal position near the cytochrome *f* heme (35). The cytochrome *f* protein, encoded by *petA*, has a membrane spanning α -helix, and associates with the side of cytochrome *b₆* core opposite the Rieske 2Fe-2S protein. A *c*-type heme is bound to the luminal, amino-terminal ‘head’ region of cytochrome *f*.

Electrons in the cytochrome *bf* high-potential domain pass from the plastoquinol-oxidation (Q_p) site through the Rieske 2Fe-2S cluster to cytochrome *f* and then to a soluble plastocyanin or cytochrome c_6 protein. Four small, hydrophobic proteins, PetG, L, M, and N, are bound to the cytochrome *bf* complexes of cyanobacteria and chloroplasts (18, 35, 38). The *petGL* genes are essential in cyanobacteria and their disruption results in decreased synthesis of the cytochrome *bf* complex in the eukaryote *Chlamydomonas reinhardtii* (39). Another apparent small subunit, PetO, was found to be phosphorylated during light harvesting protein redistribution in *C. reinhardtii*, but is apparently not present in cyanobacteria (40). The PetN was found to be essential in chloroplasts but its function in cyanobacteria is not clear (41).

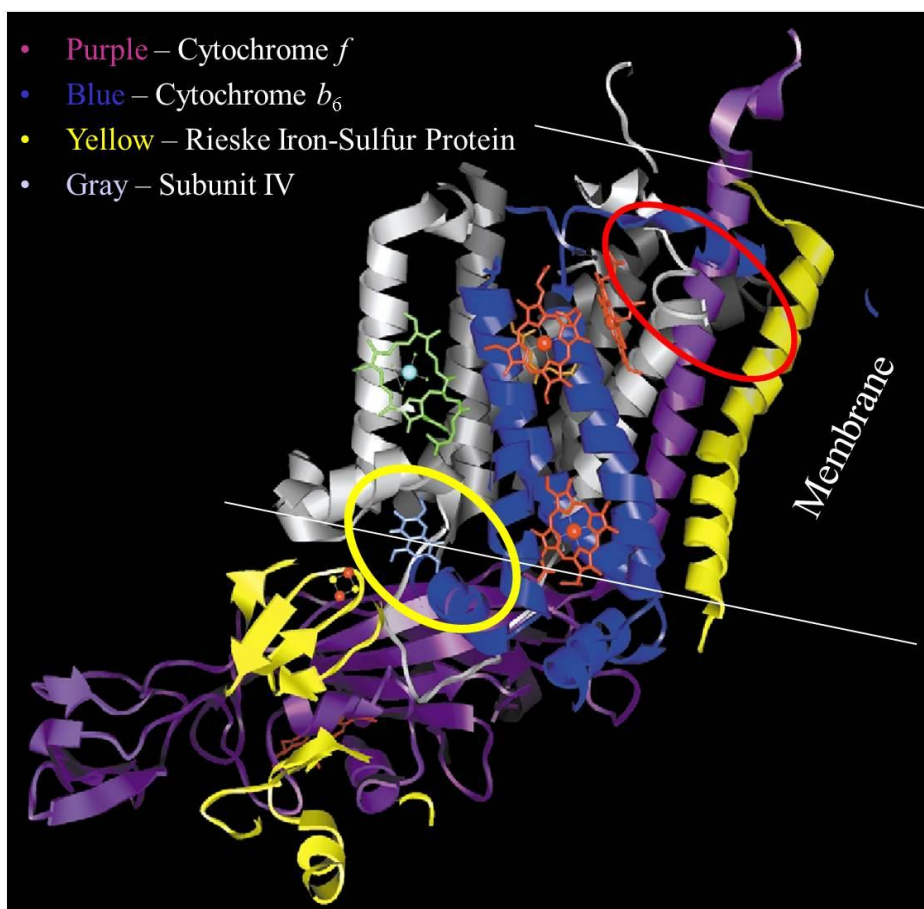


Figure. 4 – Structure of monomeric *bf* complex illustrating the protein composition. The four primary subunits making up the cytochrome *bf* complex are cytochrome *f*, cytochrome *b*₆, the Rieske Iron-Sulfur protein, and Subunit IV. Hemes are colored in red, chlorophyll in green, and the quinol oxidase site (yellow circle) is represented by a bound stigmatellin molecule in light blue. The quinone reductase site is identified by the red circle. Structure is *Chlamydomonas reinhardtii* cytochrome *bf* structure (Strobel et al., 2003) as illustrated by Allen 2004.

The pigments and hemes associated with the cytochrome *bf* complex have been resolved via X-ray crystallography (36, 35). The *b*_n and *b*_p hemes bound to the cytochrome *b*₆ protein are reduced in response to plastoquinol oxidation at the quinol oxidation (*Q*_p) site (42, 43, 41). These, together with the recently discovered *c*_n heme (36, 35), comprise the low potential chain of the cytochrome *bf* complex. This low-potential chain conducts electrons from plastoquinol at the quinol-oxidation site via the *b*_p (low-potential) and *b*_n (high-potential) hemes and the *c*_n heme to the quinone-reduction (*Q*_n or *Q*_n) site on the negative side of the membrane. The *c*_n heme is covalently bound to Cys₂₅ (numbered according to *Chlamydomonas*) of the cytochrome *b*₆ protein (35, 36).

Many functions have been proposed for the chlorophyll *a* and β -carotene molecules stoichiometrically isolated (44) and structurally integrated (36, 35) with the cytochrome *bf* complex. Chlorophyll *a* and β -carotene are photoreactive and chlorophyll *a* participates in triplet-state, excitation-mediated production of singlet O_2 (45). β -carotene functions in photoprotective grounding of singlet O_2 . Thus, β -carotene can reduce the rate of singlet O_2 produced by excited chlorophyll *a*. These two pigments are often found in close association, presumably for this reason (45). In the cytochrome *bf* complex these pigments appear to be too far apart (14 Å) to produce this photochemical quenching of chlorophyll *a* by β -carotene. The chlorophyll *a* and β -carotene molecules of the cytochrome *bf* complex have also been shown to undergo a red-shift in their absorbance peaks in response to chemical reduction of the cytochrome *bf* complex (46), implicating them in an electron-transport-based function.

The many structural components of the cytochrome *bf* complex lend themselves to many possible functions. This complex translocates protons, catalyzes bifurcated electron flow via high- and low-potential pathways, oxidizes and reduces quinol/quinone molecules, has photoreactive potential via its chlorophyll and carotenoid molecules, produces superoxide radicals, and mediates linear and cyclic electron flow. In addition, the *bf* complex is implicated in plastoquinone-pool redox-sensing and signaling that regulates photosynthesis via light-harvesting protein redistribution and regulation of gene/protein expression. The regulatory dynamics of these many functions is the primary interest and focus of this thesis.

1.2.4 - Function of the Cytochrome *bf* Complex.

The cytochrome *bf* complex is the main site of plastoquinol (PQH₂) oxidation in photosynthesis (16, 47, 36 for reviews). The *bf* complex links electron flow between the two photochemical reaction centers of photosynthesis and uses their harvested light energy to generate the proton motive force. The cytochrome *bf* complex oxidizes the quinone pool through its quinol oxidation site located near the positive (P) side of the membrane. Here the electron transport chain bifurcates with one electron from PQH₂ passing into the high potential chain and the second into the low potential chain.

The cytochrome *bf* high-potential chain (Figure 2 – in blue) consists of a series of soluble electron-conducting proteins: the Rieske Iron-Sulfur protein (ISP), cytochrome *f*, and cytochrome *c*₆ or plastocyanin. The initial oxidation of plastoquinol by the Rieske ISP transfers one electron (47) to the *c*-type heme of cytochrome *f*, which in turn transfers this electron to a lumen- or p-side soluble electron carrier, cytochrome *c*₆ or plastocyanin. This soluble electron carrier diffuses freely within the aqueous lumen space and transfers its electron to the reaction center of PS I.

The low-potential chain of the cytochrome *bf* complex (Figure 2 – in red) runs through the membrane-spanning regions of the cytochrome *b*₆ and subunit IV polypeptides, to the quinone-reductase site near the cytoplasmic- (stromal) or n-side of the membrane. Two histidine-bound *b*-type hemes of the cytochrome *b*₆ protein, *b*_p and *b*_n, conduct one electron from the p-side quinol oxidation site to the *c*-type heme, *c*_n, near the n-side of the membrane (36, 35). Electrons flowing through the low-potential chain ultimately reduce plastoquinone to plastoquinol at the n-side quinone-reductase site. It

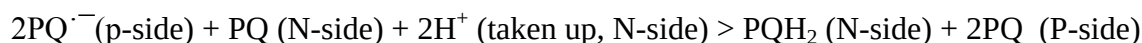
seems likely that one electron is held by the c_n heme until a second quinol oxidation event occurs sending a second electron into the low-potential chain and resulting in simultaneous, two-electron reduction of plastoquinone to plastoquinol (PQH_2) (48, 34, 29,). Alternatively, it remains possible that the first turnover of the low-potential chain generates a semiquinone ($PQ^{\cdot-}$), a plastoquinone molecule with an unpaired electron) at the quinone reductase site as in cytochrome *bc* complexes (34). The overall electron-transfer and proton-translocation mechanism of the cytochrome *bf* complex can be described in terms of a modified Q-cycle (18, 29) as summarized below.

Step 1: Plastoquinol oxidation at the quinol-oxidation (Q_p) site.



Oxidation of two plastoquinol (PQH_2) yields $4H^+$ deposited on the P-side of the membrane. Two electrons, one each from the initial oxidation of two PQH_2 molecules, pass through the high potential chain to PS I. The second electron from each PQH_2 oxidation remains transiently at the quinol-oxidation site as a reactive semiquinone radical ($PQ^{\cdot-}$).

Step 2: Plastoquinone reduction (PQ) to plastoquinol (PQH_2) at the quinone-reductase (Q_n) site.



The semi-quinone radicals ($PQ^{\cdot-}$) produced at the P-side, quinol-oxidation site in Step 1 are used to reduce a plastoquinone (plastoquinone) (PQ) to plastoquinol (PQH_2) at the

quinone-reductase site near the N-side of the membrane. Two H^+ are taken up from the N-side of the membrane in this process.

The Overall Equation:



Because PQH_2 is regenerated at the quinone-reductase site, the net reaction can be represented as:



For every two plastoquinol (PQH_2) molecules oxidized at the cytochrome *bf* quinol-oxidation site, two electrons flow through the high-potential chain to cytochrome c_6 or plastocyanin, and a minimum of four protons are deposited into the lumenal- or P -side space of the membrane. Note that every reduced plastoquinone molecule (i.e. plastoquinol) in the plastoquinone pool has already picked up two protons from the cytoplasmic- or N -side of the membrane (either from the photosystem II quinone_B site or the cytochrome *bf* quinone-reductase site). Therefore the cytochrome *bf* complex is an extremely efficient site for converting the energy from electron transport into a transmembrane proton gradient, with a minimum stoichiometry of two H^+ translocated across the membrane for each electron passed through the high-potential chain.

The existence of the plastosemiquinone ($PQ^{\cdot-}$) at the cytochrome *bf* quinol-oxidation site is of great interest for reactive oxygen species production because the

midpoint potential of the reactive semi-quinone (-300 mV or lower) is sufficiently low to pass its unpaired electron to molecular oxygen, forming superoxide ($\text{O}_2^{\cdot-}$).

1.2.5 – Cyclic Electron Transport.

Cyclic electron transport represents the “cycling” of electrons around photosystem I (PS I) via ferredoxin and the cytochrome *bf* complex (49, 50). This process has long been known yet the mechanism has remained elusive because measurements of cyclic electron are problematic (51). Such measurements require that PS II be shut down and PS I remains active. A principal difficulty is that no readily measurable substrate is consumed nor reduced product formed. Cyclic electron transport serves to regenerate reduced plastoquinone (PQH_2) in the plastoquinone pool and results in formation of proton-motive force and ATP but no reductant and thereby shifts the ATP/NADPH ratio in favor of ATP (50, 49). Cyclic electron flow might occur via a direct ferredoxin or ferredoxin-NADPH-oxidoreductase mediated delivery of electrons to the quinone reductase (Q_n) site of the cytochrome *bf* complex. Alternatively or in addition, cyclic flow from photosystem I may occur via electron transfer from NADPH to NADPH-oxidoreductase or indirectly via succinate dehydrogenase resulting in reduction of the plastoquinone pool. Several lines of evidence support such pathways (52, 53, 54). Because all of these possible cyclic pathways require the cytochrome *bf* complex at some stage in the cycle, it becomes difficult to dissect the relative contributions of these pathways. Consistent with the role of the *bf* complex in cyclic electron flow, inhibitors of the cytochrome *bf* complex largely inhibit cyclic electron transport (55, 56).

Much attention has been given to the well-known, yet largely enigmatic, inhibitor of cyclic electron transport, Antimycin A (antimycin). This inhibitor is known to bind to the quinone-reductase (Q_n) site of the cytochrome *bc* complex, the mitochondrial respiratory electron transport complex equivalent of the cytochrome *bf* complex. The *bf* complex is insensitive to antimycin and the complex has not been shown to bind the inhibitor. Interestingly, antimycin is reported to partially inhibit cyclic electron transport in cyanobacteria that lack mitochondrial respiratory chains (50, 54). A yet to be identified ferredoxin-quinone reductase has been suggested to explain this inhibition by antimycin (51). Antimycin blocks residual cyclic flow in cyanobacterial mutants defective in the *ndhB* (57) or PS I *psaE* genes (53), as well as in a chloroplast *ndhB* mutant (52). *NdhB* encodes a subunit of NADH dehydrogenase, which in cyanobacteria also likely functions as an NADPH dehydrogenase. These findings support the idea that one important pathway of cyclic electron flow uses NADH dehydrogenase as mentioned above. Thus, there seem to be at least two pathways for cyclic electron transport, one involving NADH dehydrogenase and another that is sensitive to antimycin. As many as four different pathways of cyclic electron transport or as few as one have been suggested in plant chloroplasts (49). The cytochrome *bf* quinone-reductase (Q_n) site inhibitors NQNO and HQNO were compared against antimycin relative to proton translocation and *b*-heme reduction (56). This study showed that all three inhibitors inhibited proton translocation but only NQNO and HQNO (those specific to the Q_n site) inhibited *b*-heme oxidation kinetics (56). Thus antimycin activity seems to be connected to cyclic electron transport and the cytochrome *bf* complex, perhaps involving the Q_n site, but probably not directly

as evidenced by normal *b*-heme redox kinetics in the presence of antimycin. The PGR5 protein of *Arabidopsis thaliana* was initially thought to be involved in cyclic electron transport as a component of an antimycin-sensitive pathway (51). However it turned out that PGR5 is more closely involved in non-photochemical quenching mechanisms (50). Thus, the question of cyclic electron transport, its part in ROS and redox regulation, and the role of the inhibitor, antimycin A, remain largely mysterious. Understanding these connections would greatly advance the field. Work in this thesis has attempted to address some of these questions.

Recently, a membrane spanning, multi-domain regulatory complex known as FesM has entered the picture. *Synechococcus* 7002 *fesM* null mutants cannot grow photoheterotrophically in the presence of DCMU (dichloromethyl-urea, which blocks reduction of the plastoquinone pool by PSII) presumably because of impaired cyclic electron transport (58). The mutant was determined to be impaired in cyclic electron transport because it displayed slower P700 re-reduction rates and the plastoquinone pool was more slowly reduced in the mutant compared to the wild type. Thus PS I could not be reduced as quickly in the mutant compared to the wild type, presumably because of insufficient cyclic flow. Further characterization of the FesM protein may provide insight into redox regulation and/or cyclic electron transport because the protein contains an Fe-S cluster and NtrC-like and cAMP receptor domains that may have regulatory roles. Interestingly, cyclic electron flow could not be restored to the FesM mutant unless the cAMP-binding domain was present (58). The FesM protein may possess the right

attributes to be a missing link between cyclic electron transport and redox sensing and signaling.

1.3 - Regulation by the Cytochrome *bf* Complex: Function and Implications of Redox-Sensing and Signaling

1.3.1 – Redox-dependent State Transitions and Redox Mediation.

In photosynthetic organisms, the state transition is the preferential shunting of harvested light energy to PS II or PS I in response to changes in light intensity or quality (18, 59, 60, 61, 15, 62). PS I and PS II are not identical in respect to capturing light energy (15). Consequently, the photosystems may run at different rates depending on the wavelength composition and intensity of light. Thus, if the rate of plastoquinone pool reduction via PS II and oxidation via PS I are unbalanced, the plastoquinone pool shifts toward a more reduced or oxidized state. Redox extremes in the plastoquinone pool have consequences. An over-oxidized plastoquinone is inefficient because PS I turns over too quickly for PS II to maintain adequate reduction. Over-reduced plastoquinone pools are dangerous because the longer reductive potential remains on low-potential electron transport components, the more likely superoxide formation will result. Thus, regulating the turnover rates of the photosystems relative to one another becomes important both for mitigating production of harmful by-products (ROS) and for maintaining efficient use of available energy in a dynamic light environment. To a large extent, the chloroplast, chlorophyll a/b or cyanobacterial, phycobilin light harvesting proteins are diffusible, photochemical complexes that may associate with either PS I or PSII. Association of

these light harvesting complexes increases the optical cross-section of the photosystem allowing more light energy to funnel into the reaction centers and thereby increasing turnover. This transient association with PS I or PS II is redox-dependent (18, 61, 15, 47). Light harvesting complexes preferentially associate with PS II when the intersystem electron transport chain (the plastoquinone pool and *bf* complex) is oxidized and with PS I when the chain is reduced.

The state transition is a dynamic a rapid response to environmental changes and is faster than transcriptional activation or protein turnover in regulating photosynthetic electron transport. State transitions occur over minute time-scales in eukaryotes (63) and seconds in cyanobacteria (15). This swift response to changing light environments makes the electron transport chain highly dynamic. The state transition mechanism constantly senses and finely tunes the rates of the plastoquinone pool oxidation by PS I and reduction by PS II through fine adjustment of light harvesting complexes between them. Under normal physiological conditions the redox state of the plastoquinone pool is unlikely to reach redox extremes. Thus, the state transition is a manifestation of a redox-dependent regulatory mechanism of photosynthetic electron transport and thus a model process by which to study redox sensing and signaling.

Emlyn-Jones et. al. (34) showed that state transitions were significantly impaired in *Synechocystis* strain PCC 6803 when the gene (*rpaC*) for a small transmembrane protein was deleted. State transitions mediated by RpaC (Regulator of Phycobilisome Association) are not involved in high-light growth environments and only seem relevant at low light intensities (61). This suggested that state transitions have no photoprotective

function. In this context and considering the integral ‘goal’ of electron transport as the establishment of the proton motive force, then why limit PS II activity by shunting energy away to PS I? However, another group found that chlorophyll-associated state transitions, mediated by the PS I PsaK1 and PsaK2 proteins, are important in high light and photo-protection (17). Vermaas et al. (64) found that removal of CtaI (a subunit of cytochrome *c* oxidase) in *Synechocystis* PCC 6803 caused death at light intensities of $<5 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ presumably because of an inability to regulate the reductive potential of the plastoquinone pool. Thus, it seems that preferential distribution of light-harvesting energy is important for photosynthetic efficiency and dissipation of excess energy. Clearly, under some circumstances, over-reduction of the plastoquinone pool can be lethal. Conflicting ideas about the purpose of state transitions highlights the possibility that these serve different purposes and that the sensing and signaling mechanisms may have many, branching components.

In *Synechococcus* PCC 7002, the PetB-R214H mutation occurs near the cytochrome *bf* quinone-reductase (Q_n) site (34). This mutation slows both electron flux through the low potential chain and turnover of the *bf* complex (34). The mutant also produces copious amounts of superoxide (27), which inhibit growth. One might think that slowing electron flow through the cytochrome *bf* complex would cause over-reduction of the plastoquinone pool and thereby increase superoxide production from PS II or NADH dehydrogenase. However, another mutation that slows electron flow through the *Synechococcus* 7002 cytochrome *bf* complex has quite different consequences. The PetC1-Δ2G mutant (35) is inhibited in electron transfer from the cytochrome *bf* Q_p -site to

cytochrome *f* because of two glycines deleted from the hinge-region of the Rieske ISP (Iron-Sulfur Protein). Overall turnover of the cytochrome *bf* complex is slowed comparably in the high-potential chain PetC1-Δ2G mutant and the low-potential chain PetB-R214H mutant. However, the PetC1-Δ2G mutant does not produce excessive amounts of superoxide compared to the wild-type nor does it grow significantly more slowly. Thus, both mutants impair electron transport but only R214H over-produces reactive oxygen species. One hypothesis to explain this is that in the low-potential chain PetB-R214H mutant the initial oxidation of plastoquinol at the cytochrome *bf* Q_p-site occurs as usual but the second electron transfer step is slowed because of impeded electron transfer into the low-potential chain. This prolongs the lifetime of the reactive semi-quinone (2PQ^{•-}) at the Q_p-site and thereby increases the probability of reducing O₂ to superoxide. Interestingly the PetB-R214H and PetC1-Δ2G mutants also exhibit aberrant state transitions. Thus impaired electron flux through the cytochrome *bf* complex and altered mediation of photosynthetic electron transport between PS II and PS I, has interesting implications for both ROS production and redox sensing and signaling.

1.3.2 – The Cytochrome *bf* Complex and Signaling of State Transitions in Eukaryotes versus Cyanobacteria.

In eukaryotic organisms, such as *Chlamydomonas reinhardtii*, state transitions occur via phosphorylation and probably PetO (40), a protein associated with the cytochrome *bf* complex, in response to plastoquinone binding at the Q_p site (63). Also in *C. reinhardtii*, membrane-bound chlorophyll *a/b* light harvesting complexes show redox-dependent phosphorylation patterns corresponding to state transitions (63). Several lines

of evidence indicate that state 2 (PQ pool reduced) to state 1 (PQ pool oxidized) transition involves a kinase dependent phosphorylation of light-harvesting chlorophyll *a/b* proteins (43, 42, 41, 63, 62). The phosphorylation/dephosphorylation state of these proteins determines their affinity for either PS I or PS II as they diffuse within the membrane. Interestingly, the cytochrome *bf* complex also was found to diffuse to either PS I or PS II enriched regions upon state transitions in *C. reinhardtii* (65). In spinach, redox-dependent phosphorylation of light harvesting complexes was found to be dependent on quinol binding to the quinol oxidation site of the cytochrome *bf* complex (42). It was later discovered in *C. reinhardtii*, that the small cytochrome *bf*-associated PetO protein was also found to be phosphorylated upon state transition (40), specifically in response to binding of stigmatellin to the quinol-oxidation site (63). Thus in eukaryotic photosynthetic organisms it appears that the cytochrome *bf* complex and associated subunits regulate the redistribution of light harvest complexes with reaction centers via a phosphorylation mechanism driven by turnover or redox state of the *bf* complex.

No phosphorylation-dependent state transitions events have been reported in cyanobacteria. The light harvesting complexes in eukaryotes are rings of chlorophyll *a/b*-binding proteins bound within the thylakoids whereas cyanobacteria predominantly use a very different, soluble light-harvesting complex. These protein complexes, known as phycobilisomes, are primarily associated with PS II and arranged in rods formed of individual discs of either phycocyanin (PC) or both phycocyanin and phycoerythrin (PE) (15, 66). These pigments greatly increase the optical cross-section of PS II and are thought to diffuse between the photosystems (61) in response to light intensity or quality

and the redox state of the electron transport chain. Another model for state transitions in cyanobacteria (18) suggests that the phycobilins do not diffuse between PS I and PS II but rather a redox-dependent conformational change allows the PSII-attached phycobilins to transfer their harvested light energy to PS I. Phycobilisomes have been known to change their PC:PE composition in response to changes in temperature, light intensity or wavelength (66). In *Synechocystis* 6803, deletion of the *rpaC* gene makes the organisms incapable of performing state transitions (34) as does an NADH dehydrogenase *ndhB* mutant (57). Comparing these two mutants, one finds that they are state transitions mutants by different mechanisms. The *rpaC* mutant likely involves a disruption of the redox sensing-signaling mechanism whereas the *ndhB* mutant displays a more oxidized plastoquinone pool that affects state transitions indirectly. Thus, there are indirect and direct effects to consider when assessing the induction and impacts of state transitions.

In summary, electron transport and thus ROS production, are balanced and regulated through redox sensing-signaling and state transitions. However, essential pieces to this puzzle are missing, particularly in cyanobacteria. It is still not well understood how redox levels in electron transport chains are sensed and how these signals are transmitted to elicit state-transitions or redox-dependent transcription or protein regulation events. It has become clear, however, from studies with electron transport chain inhibitors and evaluation of state transitions that the cytochrome *bf* complex plays a central role both in chloroplasts and globally important cyanobacteria (62). The overarching objective of this thesis was to gain insight into the mechanism of redox regulation by electron transfer pathways in the cytochrome *bf* complex of photosynthesis

and their relation to production of oxygen radicals. To achieve this it was necessary to understand how ROS production and redox regulation are affected by inhibitors of linear photosynthetic electron transport, cyclic electron transport, and the cytochrome *bf* Q-cycle. My goal was to provide a clearer picture of how photosynthetic energy flux and ROS production are modulated in a dynamic light environment by events within the cytochrome *bf* complex and outside of it.

1.3.3 – Hypotheses, Objectives, and Significance of this Work.

The initial hypotheses of this work were: 1) that reactive oxygen species (ROS) are primarily produced at the quinol-oxidation (Q_p) site of the cytochrome *bf* complex, 2) that the cytochrome *bf* complex is involved in regulation of state transitions (the redistribution of light-energy between the photosystems, and 3) that ROS production triggers adaptive responses through state transitions that help mitigate ROS production by subsequent adjustment of phycobilisome (light-harvesting protein) distribution.

Thus, the objectives of this work were three-fold: 1) to investigate ROS production from the cytochrome *bf* complex, 2) to determine what specific regulatory role the cytochrome *bf* complex may have in signaling state transitions (phycobilisome redistribution between the photosystems), and 3) to understand the impacts, if any, of ROS on phycobilisome redistribution.

Investigating ROS production focused on understanding the relative contributions to ROS of sources other than the cytochrome *bf* complex through measurements of ROS production in the presence of various inhibitors, inducers, and scavengers of ROS. Next, sources of ROS production from the cytochrome *bf* complex were characterized. The two

domains of quinol/quinone binding in the cytochrome *bf* complex are possible sites of superoxide production. To distinguish the contribution of these domains as sources of ROS, the inhibitors TDS (tridecylstigmatellin) and NQNO (described above) were used to occupy either the cytochrome *bf* quinol-oxidation (Q_p) or quinone-reduction (Q_n) sites and thereby exclude the potential formation of ROS-generating semiquinones from these sites.

Determining the contribution of the cytochrome *bf* complex to redox-regulated phycobilisome redistribution during state transitions also required the inhibitors NQNO and TDS. They were used to understand whether substrate binding, redox levels in the high- or low-potential chains, or other cytochrome *bf*-specific effects are involved in signaling state transitions. Lastly, understanding the impacts of ROS on redox regulation of state transitions encompassed titrations of measured state transitions under different ROS induced states. This work focused on understanding the impacts of ROS production on plastoquinone pool redox status and state transitions. The purpose of this objective was to find parameters under which ROS might affect redox sensing/signaling *in vivo* and thereby elicit a possible adaptive response. These tests of the hypotheses above are currently debated topics in the field of redox regulation in photosynthesis (18, 61). Understanding redox regulated responses to ROS production may provide a better understanding of cellular priorities and provide insight into ways in which photosynthetic cells might protect themselves from reactive oxygen species. Defining the sensing and signaling events that determine phycobilisome distribution will provide a clearer picture of the dynamic capacity of the redox sensing and signaling process in photosynthesis.

This in turn may help understand the responses of photosynthetic electron transport to environmental stresses. In the grander scope of global photosynthesis, this facilitates our knowledge about how photosynthetic microorganisms might respond to changing environments and how photosynthesis might be manipulated for purposes such as biofuels production.

Chapter 2: Material and Methods

2.1 – Strains

2.1.1 – *Synechococcus* PCC 7002.

This strain of *Synechococcus* is a marine cyanobacterium, small (~1 μm diameter) and coccus shaped. The strain contains a full complement of photosynthetic structures including PS II and PSI. It is readily transformable and its genome has been sequenced making this cyanobacterium an attractive model for genetic studies in photosynthesis. *Synechococcus* 7002 typically exhibits relatively small state transitions compared to *Synechocystis* PCC 6803.

2.1.2 – *Synechococcus* PCC 7002 PetB-R214H (R214H).

This mutant strain of *Synechococcus* 7002 is inhibited in cytochrome *bf* low-potential chain function because of the replacement of arginine 214 with a histidine in the cytochrome *b₆* protein (27, 34). The recombinant gene carries a flanking spectinomycin/streptomycin resistance marker and liquid cultures contain 50 $\mu\text{g/ml}$ of both antibiotics.

2.1.3 – *Synechococcus* PCC 7002 PetC1- $\Delta 2\text{G}$ ($\Delta 2\text{G}$).

The $\Delta 2\text{G}$ mutant carries a deletion of two glycine residues of the hinge region of the PetC1 Rieske iron-sulfur protein (RISP) (67). This impairs the ability of the Rieske ISP to transfer its electron to cytochrome *f* resulting in slower electron flux through the cytochrome *bf* high potential chain. Despite the impaired electron transfer this organism grows comparably to the wild type (WT) and does not over-produce ROS as the R214H mutation does. The recombinant gene has a flanking spectinomycin/streptomycin

resistance marker and thus liquid media are supplemented with 75 µg/ml spectinomycin to maintain selection.

2.1.4 – *Synechocystis* PCC 6803

This freshwater cyanobacterium is another model organism for genetic studies of cyanobacterial photosynthesis. Its genome was sequenced and is also easily transformable. *Synechocystis* 6803, like all cyanobacteria, carries a full complement of photosynthetic complexes (PSI and PSII) and additionally expresses quinol oxidases unlike *Synechococcus* 7002. The state transitions in *Synechocystis* 6803 are much more pronounced than in *Synechococcus* 7002, making room temperature PS II fluorescence measurements easier.

2.2 - Cultures and Growth Conditions

Stock cultures of *Synechocystis* 6803, *Synechococcus* 7002, and the 7002 mutants *petB*-R214H and *petC*-Δ2G were grown in 250 mL side-arm flasks with 125 mL of BG-11 (*Synechocystis* 6803) or A medium (*Synechococcus* 7002) supplemented, as needed, with 50µg/ml of antibiotics, at 25°C, under low light conditions (30-50 µmol photons m⁻² s⁻¹) on a shaker in ambient CO₂. As inocula for exponential phase, experimental cultures, cells were grown at their optimal growth temperatures (32°C for *Synechocystis* 6803 and 39°C for *Synechococcus* 7002) under medium light intensities (100 µM photons m⁻²s⁻¹).

2.3 - Growth Experiments

2.3.1 – Aquarium and Conditions.

When cultures were grown under specific atmospheric conditions, such as in N₂ or CO₂ gas mixtures, cells were grown in 18 mm dia. test-tubes with rubber septum tops allowing specific gases mixed via a flow meter to bubble from the bottom of the culture-tube. The aquarium was kept at a constant temperature via a heating element and water circulator. Light was provided via cool-white fluorescent light-banks at approximately 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

2.3.2 – Optical Density Measurements.

Three methods were used to determine culture growth phase and growth rates. Optical density measurements at 750 nm were performed with 1 cm and 1.8 cm light paths, respectively. Side-arm flasks and a Klett colorimeter (Klett, ca. 1945) allowed turbidity measurement of cultures directly from culture flasks. Culture density values were compared over a range of cell densities to develop an equation for conversion of Klett and absorbance units.

2.3.3 – Analysis of Growth Data.

Optical density data acquired during growth experiments were plotted over time and viewed in Microsoft Excel. Trend-lines were determined for the exponential phase of growth and doubling times were calculated from the growth rate. $T_d = 0.693/(\ln(\text{Final Cell Density}/\text{Initial Cell Density})/\Delta\text{Time}(\text{hrs}))$.

2.4 – Fluorescence Imaging with the Bio Rad FX Scanner

2.4.1 – BioRad FX Fluorescence Scanner Measurements of Reactive Oxygen Species.

A fluorescence scanner was used for imaging fluorescence from the H₂DCFDA probe (described below) from cultures in wells of microtiter plates or colonies on plates. Excitation was provided by a 488nm laser and emission measured through a 530 +/-15nm interference filter. Cells in liquid medium or colonies on plates were exposed to ambient fluorescent lighting $\sim 20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ prior to analysis in the FX scanner. Cells and plates remained in the scanner and thus in darkness unless otherwise noted. The same laser intensity and instrument settings were used throughout an experiment. However, parameters were varied among certain experiments to optimize data. Microtiter plate wells were typically loaded with 100 μL of cell suspensions unless otherwise noted.

2.4.1.1 – Cell preparation for ROS production assays. ROS was measured indirectly by the intensity of DCF fluorescence. Dihydro-dichlorofluorescein diacetate (H₂DCFDA, Invitrogen, Molecular Probes - MP 36103) is taken up by cells and cleaved within the cell to HD CF, which then reacts with ROS, preferentially with hydrogen peroxide (H₂O₂), peroxy radicals (COO $\bullet^-$), and peroxynitrite radicals(ONOO $\bullet^-$). The resulting DCF fluoresces maximally at 530nm upon excitation at 488nm (68). H₂DCFDA was added to a final concentration of 50 μM . Cells were washed three times with A-medium base or BG-11-medium base to remove metal ions that would otherwise catalyze HD CF conversion and fluorescence.

2.4.2 - BioRad FX Fluorescence Scanner Measurements of PS II

Fluorescence.

FX fluorescence scanner was also used to record relative levels of PS II fluorescence both from colonies on plates and cultures in wells of microtiter plates. Phycobilisomes associate with PS II or PS I in response to changes in the redox status of the electron transport chain. Some fluorescence measurements used excitation of phycobilisomes at 532 nm to compare changes in PS II fluorescence emission, viewed through a narrow bandpass filter, 680nm $\pm$ 5nm. This excitation and emission configuration provides information on the relative association of phycobilisomes with PSII. This process is termed phycobilisome-excitation-mediated PS II fluorescence and was used to understand state transitions and phycobilisome distribution. Samples were prepared in cool-white fluorescent lighting $\sim 20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and typically incubated at 30-50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

2.4.2.1 - State Transitions measured with the BioRad FX Scanner. For state transition measurements with the fluorescence scanner, data were acquired as described above (2.4.2). After scanning an image of the microtiter plate or colonies on plates, the sample tray was pulled out and a flexible fluorescent desk lamp (~ 30 -50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) was suspended over the samples for 3-7 minutes. The samples tray was then placed back in the instrument and another image was taken immediately. The scanning process required some 15 seconds under standard imaging settings (200 μm resolution) for a 1.1 megabyte image file. In any experiment performed using the microtiter-plate method, no more than two twelve-well rows were used and the plate was arranged such

that the scanning of the image proceeded from the first row to the second. This provided the best experimental consistency. Including more rows increased the time from incubation to imaging such that fluorescence changes in control samples in different rows became significantly different.

2.4.3 – Software and Analysis of Fluorescence Data from the BioRad FX Scanner.

Images of cell suspensions in microtiter plates were analyzed with BioRad QuantityOne Software. A standard sized, rectangular, sampling region was created within each of the wells in the image. Volumetric reports in the software subsequently reported the raw data counts, reflecting fluorescence intensity within each standard sampling rectangle. These reports were then exported in PDF format and pasted into a Microsoft Excel file for analysis. This process was performed for each image in a series of images taken over the course of an experiment. Unless otherwise noted, the composite data from these studies are from triplicate samples. The resulting graphs show phycobilisome-excitation-mediated PS II fluorescence or DCF fluorescence over time. Normalization of different data traces to a specific reference point entailed adding or subtracting the difference relative to that point from all other data points in traces to be normalized. Normalized traces are noted specifically in figure legends.

2.5 – Inhibitors and Probes

Inhibitors were stored at appropriate temperatures and conditions as noted in Table 2 until ready for use. The inhibitors used were NQNO (2-n-nonyl-4-hydroxyquinoline

N-oxide), TDS (tridecyl stigmatellin), KCN (potassium cyanide), malonate, DBMIB (dibromomethylisopropyl benzoquinone), DMSO (dimethyl sulfoxide), H₂DCFDA (2',7'-dichlorodihydrofluorescein diacetate), DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), methyl viologen (1,1'-dimethyl-4,4'-bipyridinium dichloride), and FCCP (carbonylcyanide-p-trifluoromethoxyphenylhydrazone). As

Table 2. – Inhibitors and Reagents.

Reagent	[Stock]	[Final]	Comments	Storage Conditions	Solvent
NQNO (2-n-nonyl-4-hydroxyquinoline N-oxide)	6.4 mM	1-10µM	Binds Qi site of Cyt <i>bf</i> complex	-20°C, Protect from Light	EtOH
TDS (tridecyl stigmatellin)	11.1 mM	10-40µM	Binds Qo site of Cyt <i>bf</i> complex	-20°C	EtOH
KCN (potassium cyanide)	50mM	50-200µM	Chelates metal ions (hemes)	25°C	H ₂ O
DBMIB (dibromomethylisopropyl benzoquinone)	10mM	0.1-20µM	May bind PSII Q _A > 20 µM	-20°C, Protect from Light	EtOH
H ₂ DCFDA (2',7'-dichlorodihydrofluorescein diacetate)	10mM	10-100µM	Fluorescence 430 nm	-20°C, Protect from Light	EtOH
DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea)	50mM	10-50µM	Binds QA site of PSII	-20°C	EtOH
DMSO (dimethyl sulfoxide)	10% w/v	1% v/v	ROS Scavenger	-20°C	H ₂ O
FCCP (carbonylcyanide-p-trifluoromethoxyphenylhydrazone)	1mM	1-10µM	Ionophoric acid between 6-8 pH	-20°C	EtOH

Stock solutions were maintained in 95% ethanol except for KCN and DMSO which were kept in water. Inhibitors were purchased from Sigma and with the exception of NQNO and TDS.

2.6 – 77K Fluorescence Emission Spectrophotometry

2.6.1 – Sample Preparation.

These experiments required incubation of cell samples with inhibitors, in the dark, in 4 mm diameter glass tubes prior to freezing in liquid nitrogen. Small diameter glass tubes closed at one end served as sample tubes for freezing cells in a liquid nitrogen dewar. Inhibitors were added to these tubes prior to the addition of cells and forced to the bottoms of the tubes by centrifugation for 5 minutes at 3700 x g in a Beckman GP centrifuge with a swinging bucket rotor. Narrow tipped pipette tips were then loaded with cells (100 µl) and capped using a sticky dot (ordinarily used for marking the tops of microcentrifuge tubes). The loaded pipette tips were placed within the tops of the glass

tubes. This allowed the cell suspensions to remain separated from any inhibitors that may have remained on the sides of the glass test-tubes. The glass tubes with the loaded pipette tips were placed into the centrifuge and incubated in the dark for 15 minutes. The sticky caps were then removed under cover of darkness to allow the cell suspensions to flow from the pipette tips into the glass tubes. The tubes, still in darkness, were then centrifuged briefly to force the cells to the bottoms of the tubes to mix with the inhibitors placed inside previously. The tubes were allowed to incubate in the dark for another 5 minutes, at which time the samples destined for freezing in darkness were frozen in liquid nitrogen in the dark. If necessary, a long-wave-length UV hand-lamp was used sparingly to provide light to operate safely. Samples destined for freezing after exposure to light were then incubated under growth-light intensity fluorescent light ($30\text{-}50\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$) for 3 minutes and then frozen in liquid nitrogen.

2.6.1.1 – Chlorophyll Determination. Chlorophyll was measured according to the Porra method by adding 50 μl of cells to 950 μl of methanol. This mixture was allowed to stand for 2 minutes and then centrifuged for 2 minutes at 14,000 rpm. The absorbance of the supernatant was recorded at 652, 665.2, and 750 nm in an Agilent 8453 UV-visible spectrophotometer. Chlorophyll concentration, in $\mu\text{g/ml}$, was calculated with the following equation: $16.29 \times (\text{OD}_{665.2} - \text{OD}_{750}) - 8.54 \times (\text{OD}_{652} - \text{OD}_{750}) \times \text{dilution factor}$ (27).

2.6.2 – Fluorescence Emission Spectrophotometry.

The purpose of this method was to view the relative fluorescence emission intensities of phycobilisomes ($\sim 660\text{ nm}$), PS II ($\sim 685\text{ nm}$), and PS I ($\sim 720\text{ nm}$) upon excitation of phycobilisomes with 580 nm light.

2.6.2.1 – Apparatus. A PTI (Photon Technologies Inc.) xenon arc lamp supplied the excitation light through adjustable bilateral slits to a chamber where the sample was suspended. The fluorescence from samples was collected perpendicularly to the path of excitation through a 610 nm long-pass filter (blocks light of 610nm and shorter wavelengths) and collected by a photomultiplier. A specialized dewar designed for spectral fluorescence analysis of frozen samples was filled with liquid nitrogen and used to hold the sample tubes. These slender tubes were also filled with liquid nitrogen to keep the samples frozen during excitation. The dewar was suspended in the sample chamber by means of a ring-stand and adjustable clamps. A lead brick was used to keep the ring-stand in place. The dewar was adjusted such that the excitation light beam impinged directly on the sample in the dewar the right angle fluorescence exited through the 610 nm long-pass filter to the photomultiplier. Once the dewar position was set it was not adjusted for the remainder of the experiment. An additional sample had been frozen for the purpose of instrument alignment and spectral calibration (described below). Nitrogen gas continually flowed into the collection chamber to reduce condensation on the dewar. A dense black cloth was used to cover the sample chamber and dewar during data acquisition.

2.6.2.2 – Data Acquisition. The FelixPTI program was used to communicate with the PTI fluorescence emission spectrophotometer. The excitation wavelength was set at 580nm, preferentially exciting phycobilisomes. Emission spectra were recorded from 620 nm to 800 nm at a sampling rate of 1 nm per 10 milliseconds. The excitation bilateral slits were set to a bandwidth approximating an excitation bandwidth of 580 +/- 5nm. The

emission bilateral slits were adjusted such that fluorescence counter did not exceed 1.5×10^6 counts and spectra were smooth with low baseline noise.

2.6.3 – Analysis of Spectra.

The raw fluorescence data were loaded into *Paul's Little Helper* spectral analysis program (P. Brantmier, visual basic, 2007, unpublished). Within this program, an equation allows a user-defined baseline to be subtracted from each spectrum. The data were then normalized to obtain equivalent amplitudes of selected fluorescence emission peaks. The program multiplies the Y-values of a selected spectrum by a user-defined ratio based on the signal amplitude of the selected spectrum relative to the signal amplitude of another spectrum at a selected wavelength. This process adjusts for variance in data collection and provides a relative, quantitative comparison of spectra. Signal intensities at 620 nm and 755 nm were chosen for the baseline because these wavelengths are outside of the wavelengths of fluorescence from phycobilisomes, PSII, and PSI. Spectra were normalized to PS I fluorescence at 720 nm to observe state transitions and to 650 nm to observe relative distributions of phycobilisomes.

Chapter 3: Results

3.1 – Growth and ROS Production in *Synechocystis* PCC 6803, *Synechococcus* PCC 7002, and Mutants PetB-R214H and PetC1-Δ2G

3.1.1 – Growth of *Synechococcus* PCC 7002 and Mutants PetB-R214H and PetC1-Δ2G.

Growth was significantly slower in the cytochrome *bf* low-potential chain mutant, PetB-R214H relative to the wild type (WT) and high-potential chain, PetC1-Δ2G mutant in which electron flow through the cytochrome *bf* complex is similarly impaired (Figure 5). R214H was found to produce more ROS than Δ2G (27) and thus the hypothesis was that increased ROS production in R214H is responsible for the reduced growth rate of R214H.

To test this hypothesis I grew wild type *Synechococcus* 7002 and the mutants R214H and Δ2G bubbled in air, 3% CO₂ or N₂, 3% CO₂ and calculated the growth rate for each which was then converted to doubling times (t_D). Doubling time is an expression used to compare the time required for the culture density to double. If R214H grew slowly because of higher rates of ROS formation then growth in N₂ would limit the oxygen available for production of superoxide and thus relieve the oxidative stress impairing the growth of R214H.

The WT and Δ2G mutant grew equally well in air, 3% CO₂ or N₂, 3% CO₂ with doubling times (t_D) of ~6 h. In contrast, R214H grew much slower in air, 3% CO₂ (t_D ~15 h) but significantly less slowly (t_D ~10 h) when bubbled with N₂, 3% CO₂ to remove

oxygen. The $\Delta 2G$ and R214H mutants are both impaired in electron flow through the cytochrome *bf* complex (27) , However, as discussed in the introduction, $\Delta 2G$ is defective in the hinge region of the Rieske iron-sulfur protein, slowing the initial oxidation of plastoquinol at the quinol oxidation site (Q_p) and electron flux in the high-potential chain. In contrast, the R214H mutation in the quinone-reductase (Q_n) domain slows electron flow in the low-potential chain and thereby slows oxidation of the plastosemiquinone radical ($PQ^{\cdot-}$) formed at the Q_p site after the initial electron transfer from plastoquinol into the high-potential chain. As discussed, this low-potential semiquinone may reduce oxygen to superoxide. The data presented here suggest that slowdown of electron flow in the cytochrome *bf* low-potential chain *per se* may not limit growth in the R214H mutant. Rather, as suggested by improved growth under N_2 , exposure to oxygen resulting in superoxide formation and ROS damage, may be the primary cause of slow growth in this mutant.

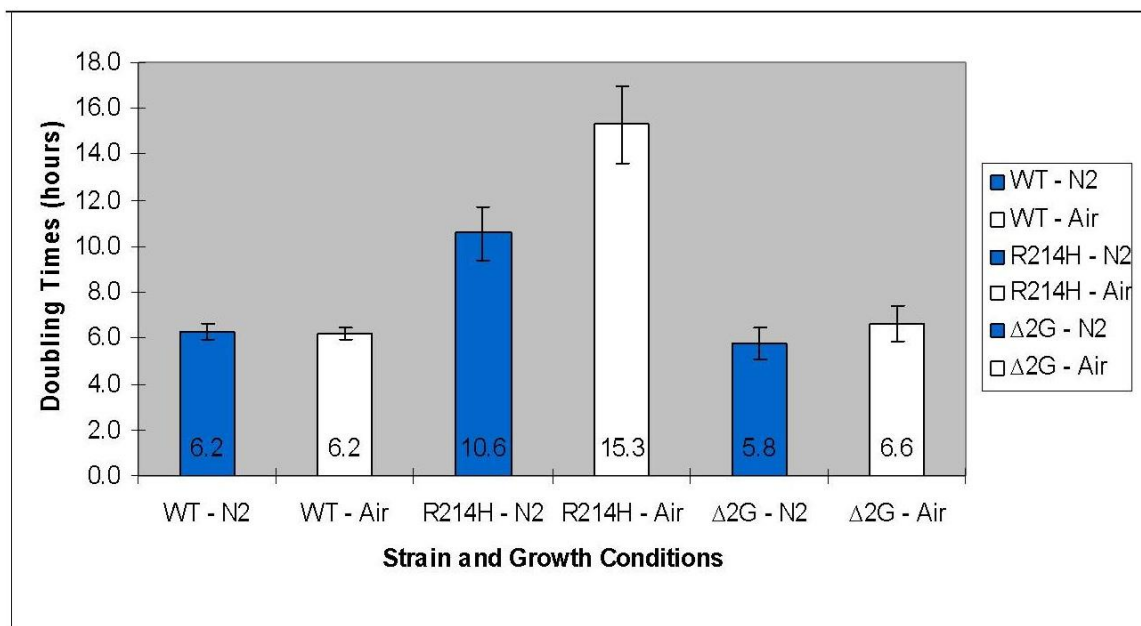


Figure. 5 – Doubling Times of *Synechococcus* sp. PCC 7002 and the mutants R214H and Δ2G in Air and N₂. Cells were grown in 10 mL test tubes at 39°C at 200 μmols of Photons/m²/s⁻¹ with individual sterile supplies of 3% CO₂, and Air, or N₂ (to displace oxygen). Doubling times were calculated using optical density measurements taken as described in Materials and Methods section 2.3 during exponential growth phase.

3.1.2 – Reactive Oxygen Species Production in the Cytochrome *bf* Low-Potential Chain, PetB-R214H Mutant Compared to the Wild Type.

To further characterize the nature of oxygen radical formation in the PetB-R214H low-potential chain mutant it was important to use the fluorescent probe, H₂DCFDA, to detect ROS. Previous studies by Darryl Horn (27) showed that the low-potential chain, PetB-R214H mutant produced ROS at high rates relative to the high potential chain mutant PetC1-Δ2G, as measured by calculating the increase in absorbance of a superoxide-sensitive XTT-formazan (3'-[1-[(phenylamino)carbonyl]-3,4-tetrazolium]bis(4-methoxy-6-nitro)). The hypothesis was that fluorescence from H₂DCFDA in response to ROS would show higher ROS production in the R214H mutant

than the WT. This experiment would ensure that the relatively non-specific ROS detection by H₂DCFDA can be used to measure forms of ROS produced by the R214H mutation in agreement with the superoxide-specific data collected using XTT (27).

The low-potential chain mutant, PetB-R214H produced ROS at rates at least five times greater than in the wild type (determined by dividing the trendline rate of R214H by that of the wild type) when incubated in darkness with glycerol (Figure. 6). As mentioned above, in cyanobacteria the plastoquinone pool becomes reduced in darkness because of electron flow from succinate and NADH dehydrogenases. In *Synechococcus* 7002, glycerol provides a source of electrons for this. These data obtained with the fluorescent probe, H₂DCFDA, corroborate previous studies performed with the optical probe, XTT, to measure superoxide production (27). Thus, the relatively non-specific ROS probe H₂DCFDA and the superoxide-specific probes are in agreement that ROS production was considerably higher in the PetB-R214H mutant than in the wild type. However, rates measured with XTT were greater than those reported here for H₂DCFDA. The increase of ROS production in R214H over the wild type over a sixty minute period appears significant.

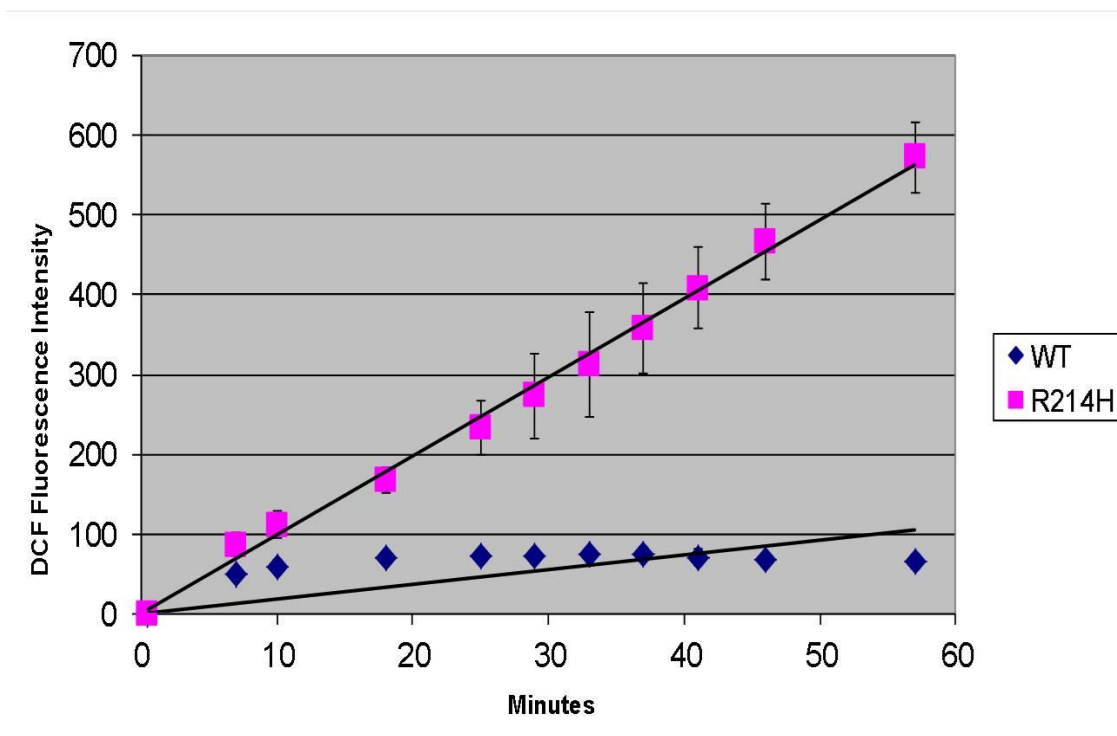


Figure. 6 – Increased ROS production in *Synechococcus* PetB-R214H relative to wild type. ROS production was measured over time from cultures of R214H and WT by H₂DCFDA oxidation and DCF Fluorescence. The BioRAD FX Scanner was used to measure fluorescence (530 nm) from the wells of microtiter plates containing 100 μ L aliquots of culture with H₂DCFDA added to a final concentration of 100 μ M. Glycerol was present (0.2%) to promote electron flow while in the dark cultures were grown in A medium as described in Materials and Methods section 2.2.

3.1.3 – ROS production in *Synechocystis* PCC 6803, *Synechococcus* PCC 7002, and the *Synechococcus* cytochrome *bf* low-potential chain, PetB-R214H mutant in the presence of inhibitors.

The R214H mutation slows electron transfer through the low-potential domain of the cytochrome *bf* complex (34). NQNO is an inhibitor of the quinone-reductase (Q_n) site of the cytochrome *bf* complex and has been shown to increase reduction and impede the oxidation of the *b* hemes in a manner similar to that of the R214H mutation (34).

Therefore NQNO was used to further test the impact of impaired electron flow in the

cytochrome *bf* low-potential chain on production of reactive oxygen species. The rates of ROS were also compared in light and darkness to determine whether the sources of R214H and NQNO-mediated ROS production were dependent on light. The hypotheses were that: 1) NQNO would increase ROS production in the wild type but not in R214H and 2) illumination would increase ROS formation in all samples.

ROS production was measured in the R214H mutant and wild type in the presence and absence of NQNO and either in cells in the dark or exposed to light ($\sim 50 \mu\text{mol}$ of photons $\text{m}^{-2} \text{s}^{-1}$) for ten minutes (Figure 7). NQNO at $30 \mu\text{M}$ provided inhibition of the quinone-reductase (Q_n) site without fear of secondary effects on the plastoquinone pool by inhibition of a quinol oxidase because these complexes are absent in *Synechococcus* 7002 (32). ROS production increased in both the wild type and R214H mutant after treatment with NQNO by a factor of 2.7 and 2.3 respectively (Figure 7). It further seems that the R214H mutation and NQNO act differently within the cytochrome *bf* Q_n site because NQNO addition did not lead to the same level of ROS formation in the wild type as in the R214H mutant.

The increase in the ROS production rate after illumination in the wild type (treated with NQNO) is much larger (3.5 times greater) than that observed in the R214H mutant (treated with NQNO) (1.8 times greater). This further suggests that the R214H mutation and NQNO may act differently with respect to their sites of inhibition for ROS production. Previously it was suggested that inhibition of *b* heme oxidation slows cytochrome *bf* turnover and increases ROS production because the reactive plastosemiquinone generated at the Q_p -site can no longer readily discharge its electron

into the cytochrome *bf* low-potential chain (27). It was suggested that this increases the Q_p -plastoquinone and thus the probability of superoxide generation. Data shown here indicate that NQNO and the R214H mutation have, to some extent, overlapping but different sites of action for ROS production relative to turnover of the cytochrome *bf* complex. These data are consistent with the conclusion that impaired *b*-heme oxidation in the R214H mutant and NQNO treatment have a common mode-of-action in that they both increase ROS production by slowing electron flow in the cytochrome *bf* low-potential chain. That NQNO-treatment of the wild type did not generate the same level of ROS as that observed in the R214H mutant suggests: 1) that the greater ROS production in R214H was due to more than simply inhibition of the *b* hemes or 2) that the R214H mutation inhibits *b*-heme oxidation more completely than NQNO does. This will be discussed further in the discussion section.

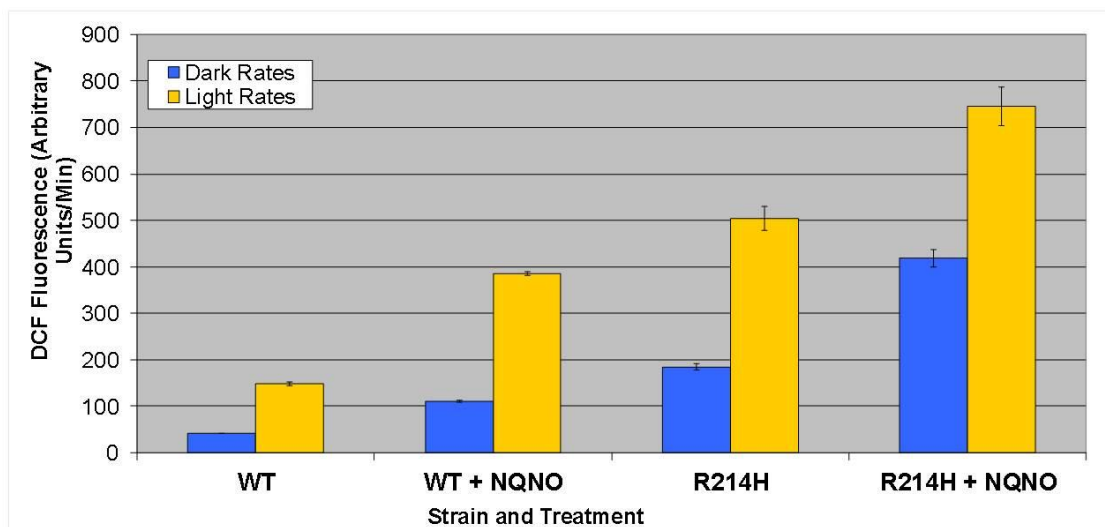


Figure. 7 – Light and Dark rates of ROS production in NQNO-treated *Synechococcus* wild type and PetB-R214H. ROS production was measured in the dark and in the light over time by DCF fluorescence from cultures of R214H and WT, treated with NQNO or not treated. The Bio RAD FX Scanner was used to measure fluorescence (530 nm) from the wells of microtiter plates containing 100 μ L aliquots of culture with H_2DCFDA added to a final concentration of 100 μ M. Glycerol was present (0.2%) to promote electron flow while in the dark and cultures were grown in A medium as described in Materials and Methods section 2.2.

3.1.4 –ROS Production in *Synechocystis* PCC 6803 in the Presence of NQNO and Tridecylstigmatellin (TDS).

I investigated whether addition of TDS to NQNO treated cultures would decrease ROS production (Figure 8). When 10 μ M TDS was added together with NQNO (Figure 8), ROS production was significantly lowered in both the 0.25 and 5 μ M treatments of NQNO. Addition of 1 μ M TDS to 0.25 μ M NQNO-treated cultures inhibited ROS production as much as did the addition of 10 μ M TDS. Figure 8 shows that even a ten-fold increase in TDS concentration did not further decrease ROS production in NQNO-treated cells. This suggests that a source of ROS production exists, in the Cyt *bf* complex or upstream, that cannot be mitigated by TDS addition.

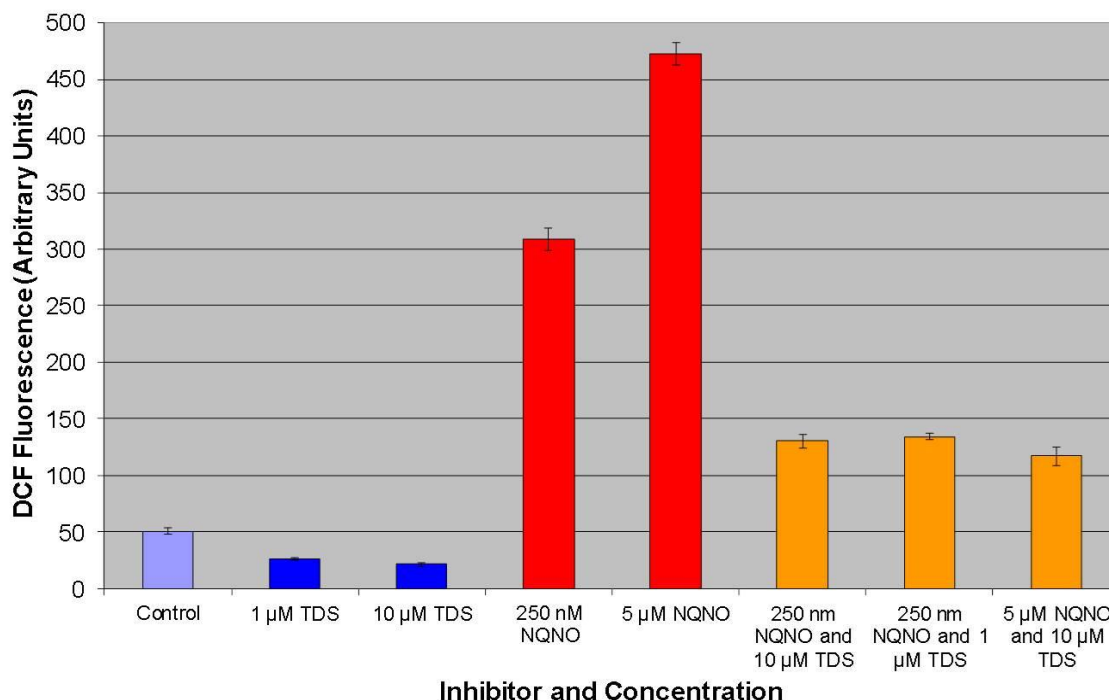


Figure. 8 – ROS production in *Synechocystis* PCC 6803 treated with NQNO then TDS. ROS production was measured from 100 μ L culture aliquots in microtiter wells containing 100 μ M H_2DCFDA . These aliquots were then not treated (control - Periwinkle), treated with TDS (1 or 10 μ M - Blue), NQNO (250 nM or 5 μ M - Red), and NQNO treated aliquots (250 nM or 5 μ M) with TDS (1 or 10 μ M – Orange). The BioRAD FX Scanner was used to measure fluorescence (530 nm) from the wells of the microtiter plates. Cultures were grown to OD_{750} of 0.41 in BG-11 as described in Materials and Methods section 2.2. The inhibitors were added in ambient room light.

3.1.5 – Reactive Oxygen Species Production in the Presence of ROS

Scavengers, Enhancers, and Inhibitors.

The reagents Methyl Viologen (1,1'-dimethyl-4,4'-bipyridinium dichloride), which enhances superoxide production from PS I in the light (122); DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), which blocks the PS II Q_B -site and thus reduction of plastoquinone pool in the light (69); and DMSO (dimethyl sulfoxide), a putative ROS scavenger (70), were chosen to probe the contribution of other sites or sources on ROS production within the electron transport chain. Cultures were treated with these reagents

in ambient room light and then measured for DCF fluorescence initially in the dark with the BioRad FX scanner (time, 0-22 min) prior to a period of light incubation (time, 25-35 min). During the initial dark incubation, there was little difference among the treatments as expected for DCMU and MV because they act only under photosynthetic conditions. After the 10 minute illumination period, the traces diverged (Figure 9). MV increased ROS production dramatically as anticipated because it removes electrons from PS I and reduces O_2 directly to superoxide. Samples containing DCMU showed a decrease in ROS production during illumination suggesting that a more oxidized plastoquinone pool and thus limiting substrate for the cytochrome *bf* complex decreased ROS production. This again implies that the cytochrome *bf* complex is an important site of ROS production under these conditions. DMSO, a putative lipophilic scavenger of ROS, also lowered ROS production implying that some of the ROS detected by DCF fluorescence arose from lipophilic sources. As mentioned in the introduction, superoxide from the cytochrome *bf* complex, as well as other sources, can lead to the subsequent formation of downstream, lipid-soluble oxygen radicals.

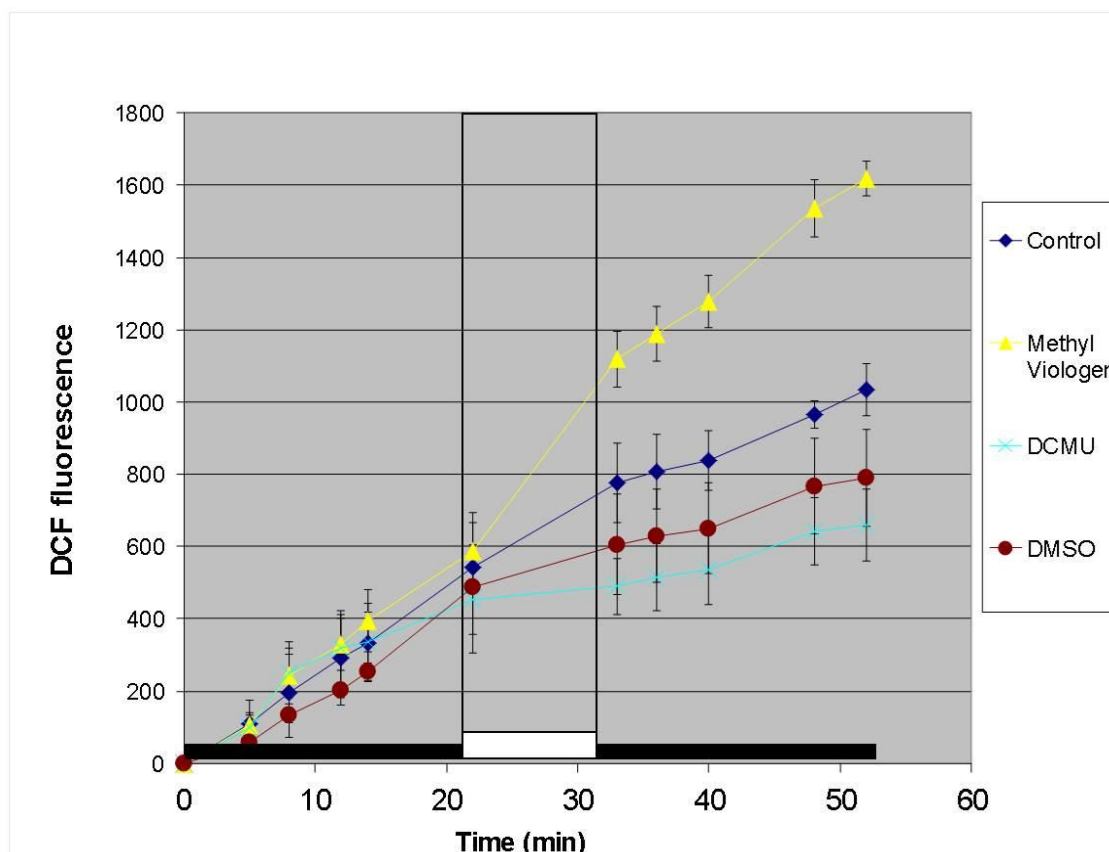


Figure. 9 – ROS production in the presence of inhibitors and ROS scavengers. ROS production was measured from 100 μ L culture aliquots in microtiter wells containing 100 μ M H₂DCFDA. These aliquots were then not treated or treated with Methyl Viologen (100 μ M), DCMU (100 μ M), or DMSO (1% v/v). The BioRAD FX Scanner was used to measure fluorescence (530 nm) from the wells of the microtiter plates. Cultures were grown to OD₇₅₀ of 0.75 in BG-11 as described in Materials and Methods section 2.2. The inhibitors were added in ambient room light prior to analysis. During the time-period indicated by a white bar on the x-axis and the box the cells were illuminated with a growth intensity light. During periods indicated by the black bar cultures remained in the dark.

3.2 – Redox Regulation of State Transitions in *Synechocystis* PCC 6803

3.2.1 – Room Temperature Photosystem II Fluorescence, State Transitions, and Estimation of the Redox Status of the Plastoquinone Pool .

At the beginning of the photosynthetic electron transport chain, PS II requires light energy to add reductive potential to the plastoquinone pool. At the end of the chain,

PS I requires light energy to oxidize the electron transport chain. PS I and PS II do not turn-over at the same rate and in order to balance the input from PS II with the output from PS I, mobile light-harvesting antennae are required. These mobile light harvesting antennae (light-harvesting chlorophyll complexes in eukaryotic photosynthetic organisms, and primarily phycobilisomes in cyanobacteria) associate with the photosystems (PS I and/or PSII) and capture additional light energy for the photosystem with which they have associated. Whether phycobilisomes associate with PS I or PS II depends on the redox status of the electron transport chain. If the electron transport chain is reduced then phycobilisomes will preferentially associate with PS I, presumably to make PS I turn-over faster and oxidize the over-reduced electron transport chain to re-establish redox balance. State transitions (described in more detail in section 1.3.1) are the preferential shunting of harvested light-energy to either PS I or PS II in response to changing light intensity or quality.

Phycobilisome-mediated excitation and PS II fluorescence was used to gauge the relative redox status of the plastoquinone pool. A high level of PS II fluorescence upon phycobilisome excitation suggests a relatively oxidized state of the plastoquinone pool. Under these conditions phycobilisomes associate preferentially with PS II thereby increasing its optical cross-section and allowing more energy to be used to reduce the plastoquinone pool (34, 18). Conversely, PS II fluorescence is low when the plastoquinone pool is highly reduced.

Tridecystigmatellin (TDS), an inhibitor of the cytochrome *bf* Q_p-site, blocks plastoquinol oxidation, and thereby causes plastoquinol (the reduced form of

plastoquinone) to accumulate in the plastoquinone pool (63). The cytochrome *bf* complex is the major pathway for plastoquinone pool oxidation (26). Thus, we can expect a more reduced plastoquinone pool and much slower turnover of the cytochrome *bf* complex in TDS-treated cultures relative to untreated samples. Similarly, NQNO is expected to reduce the plastoquinone pool. NQNO, by nature of its effect on the *b*-hemes and slowdown of the cytochrome *bf* low-potential chain, may also slow overall turnover of the *bf* complex (34, 71, 72). In addition, NQNO has been implicated in inhibition of cyanobacterial quinol oxidases and thus would further reduce the plastoquinone pool because it also blocks a secondary means of plastoquinone pool oxidation (33, 26).

3.2.1.1. – State transitions occur in *Synechocystis* PCC 6803 despite inhibition of plastoquinone pool oxidation by TDS and KCN. Initial experiments measuring state transitions showed that TDS did not impair the state transition. In fact, addition of TDS increased the amplitude of the state transition relative to the control (data not shown). Subsequent experiments investigated the hypothesis that although TDS should increase PQ pool reduction, alternative pathways such as the quinol oxidase (QOX) or plastid terminal oxidase (PTOX) actually resulted in a net oxidation of the PQ pool. Contrary to this, addition of KCN (70 μ M), an inhibitor of cytochrome oxidases, to TDS treated samples showed further enhanced state transition. This suggested that if the increased reduction of the plastoquinone pool caused by TDS was compensated by some alternative terminal oxidase, this was not sensitive to KCN. Cyanide-insensitive terminal oxidases of cyanobacteria have been postulated previously, although no evidence of this has been found in more recent studies of *Synechocystis* (33).

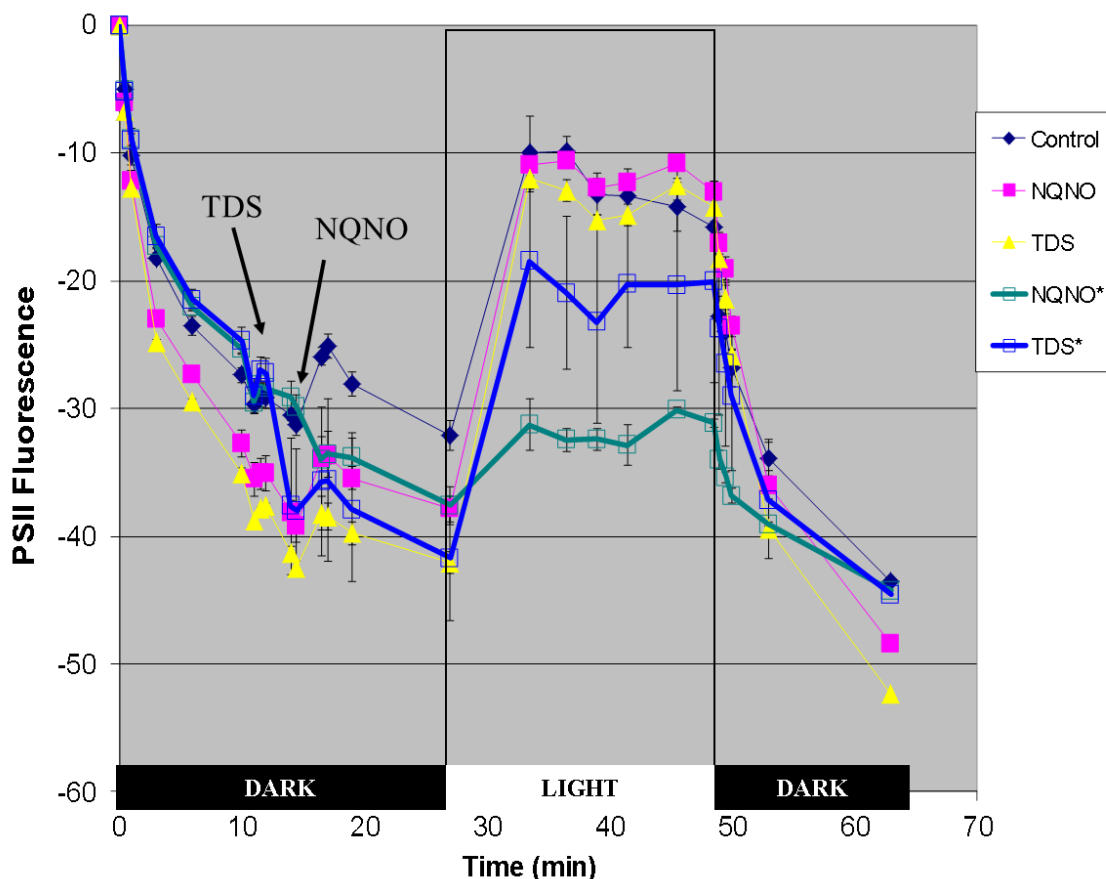


Figure. 10 - Cytochrome *bf* inhibitors and state transitions in *Synechocystis* PCC 6803. The BioRAD FX Scanner was used to measure PSII fluorescence (530 nm) from 100 μ L culture aliquots in microtiter wells containing either TDS (10 μ M) or NQNO (1 μ M) added in ambient room light or the same concentration where used to treat non-treated samples after incubation in the dark (*) at the time points indicated (Arrows. During the time-period indicated by the white bar on the x-axis and the box cells were illuminated with a growth intensity light to assess the state transition. Cultures were grown to OD₇₅₀ of 1.2 in BG-11 as described in Materials and Methods section 2.2.

3.2.1.2 - NQNO inhibits state transitions when added under reducing

conditions. Differential effects of inhibitors have been noted when these are added under either oxidizing or reducing conditions. Data presented here demonstrate that NQNO also acts differently when added under different redox poises of the plastoquinone pool. When NQNO is added to cells under growth-intensity light ($\sim 50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, thus

state 1 conditions with the PQ pool and the cytochrome *bf* low-potential chain both largely oxidized) prior to a period of dark incubation, the dark to light (state 2 to 1) state transition is not significantly impaired. However, when added under dark, reducing conditions (state 2, plastoquinone pool and cytochrome *bf* complex mostly reduced), NQNO significantly inhibited this state transition (Figure 10). Therefore I find that blockage of the Q_p site, with oxidation of the *b*-hemes, allows or even enhances the state transition whereas blockage of the Q_n site (at least in darkness), with reduction of the *b*-hemes inhibits the state transition. Overall, these data indicate that the *b*-hemes or the cytochrome *bf* low-potential chain play an important role in signaling redistribution of phycobilisomes in response to light. However, this effect of the *b*-heme redox status appears to be light-dependent because NQNO inhibited state transitions only when added to dark-adapted cells and not to cells that had been pre-incubated in the light.

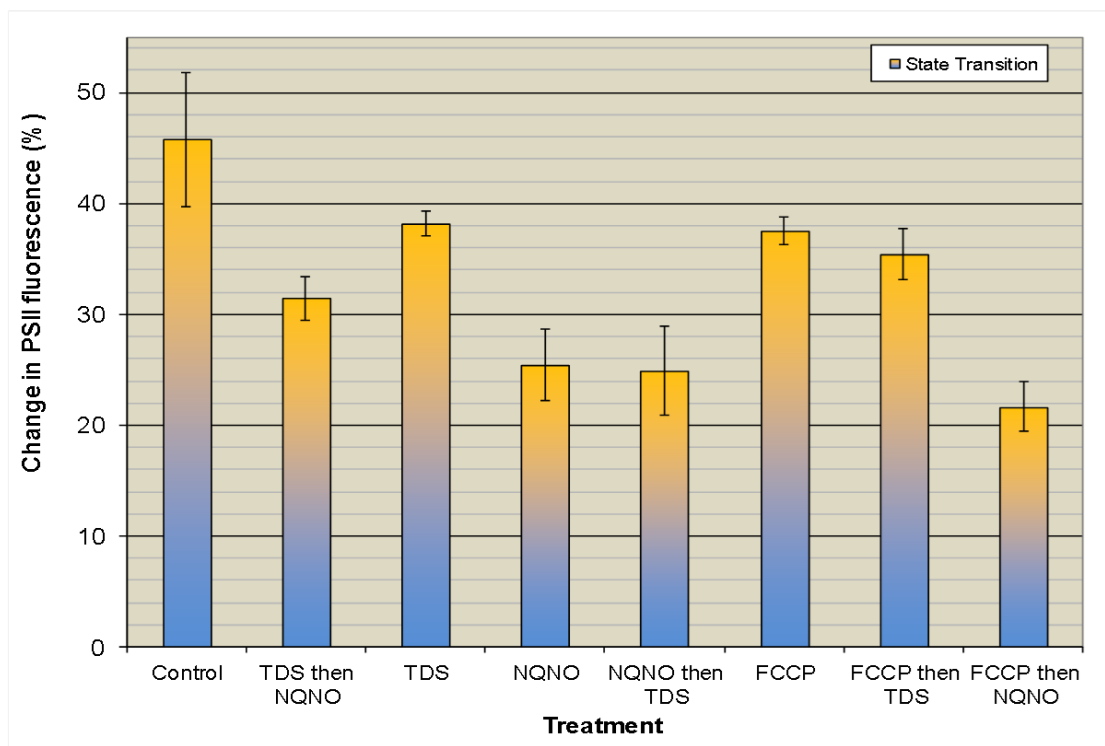


Figure. 11 - Dark to light state transitions as a function of cytochrome *bf* inhibitors and the uncoupler FCCP in *Synechocystis* PCC 6803. The BioRAD FX Scanner was used to measure PSII fluorescence (530 nm) from 100 μ L culture aliquots in microtiter wells. Cultures were treated in the dark with inhibitor NQNO (1 μ M), TDS (10 μ M), FCCP (1 μ M), or combinations indicated in the x-axis. The cells were then exposed to growth intensity light after redox poise was reached and their state transitions were measured as the relative % increase in PBS-mediated PSII. Cultures were grown to OD₇₅₀ of 1.0 in BG-11 as described in Materials and Methods section 2.2.

3.2.1.3 – Hypothesis: The redox status of the cytochrome *bf* low-potential

domain or *b*-hemes signals state transitions. Experiments focusing on the interaction of the inhibitors, TDS and NQNO, suggest that the redox status of the *b* hemes may specifically be that which is “sensed”, mediating the signaling of state transitions during transitions from darkness to light. In previous experiments (Figure 10), NQNO inhibited the state transition when added to cells in the dark but not when added in the light. Data presented above (Figure 10) further showed that TDS oxidizes both the high- and low-potential chains of the *bf* complex whether added under light (PQ oxidizing) or dark (PQ

reducing) conditions. These data show that TDS permitted the dark to light state light transition regardless of light or dark conditions. Moreover, data shown in Figure 11 show that TDS, presumably by maintaining the cytochrome *bf* low-potential chain in an oxidized state, can counteract the inhibition of state transitions by NQNO. It is unlikely that binding of TDS, at the concentration used, prevents NQNO from binding. Although Yan and Cramer (73) recently showed a secondary binding site for TDS in the Q_n -domain of cytochrome *bf* crystal structures, Alric et. al. (48) show that addition of 20 μ M TDS does not significantly impact the mid-point potential of the c_n and *b*- hemes. These data suggest that TDS does not change the binding environment of the quinone-reductase Q_n -domain near the c_n and b_n hemes. This, suggests that NQNO still binds to the Q_n -site but is unable to influence the redox potential of the *b*-hemes because the presence of TDS at the Q_p site blocks their reduction keeping the *b*-hemes oxidized. If the state transition were signaled by the redox status of the plastoquinone pool, upstream of the cytochrome *bf* complex, then TDS would inhibit the state transition, which it does not (Figure 10). Furthermore, reduction of the *b*-hemes by NQNO, and inhibition of the state transitions can be counteracted by addition of TDS, which would result in the slow, subsequent oxidation of the *b*-hemes. This is further evidence that state transitions are signaled by the redox state of the *b*-hemes and not the redox state of the plastoquinone pool upstream or redox elements downstream of the cytochrome *bf* complex.

3.2.1.4 – NQNO inhibits state transitions even in the presence of FCCP or TDS.

FCCP (carbonylcyanide-p-trifluoromethoxyphenylhydrazone), as an uncoupler and protonophore, moves hydrogen ions across the membrane in response to the transmembrane electrochemical charge gradient, effectively collapsing the proton motive force or ΔpH (74). State transitions were only slightly affected by 1 μM FCCP (Figure 11). In the presence of FCCP, NQNO continued to inhibit state transitions. These data show that state transitions in TDS or NQNO-treated cells were unaffected by addition of FCCP suggesting that the effects of TDS and NQNO on state transitions are independent of FCCP and changes in ΔpH . However, I determined subsequently that high concentrations of FCCP of 16 μM were able to match the NQNO level of State Transition inhibition (data not shown). The effect of FCCP was additive with that of NQNO, meaning that together inhibition was greater than treatment separately. Importantly, the data in Figure 11 also show that addition of TDS prior to NQNO largely prevented the NQNO-mediated inhibition of state transitions. In contrast, NQNO continued to diminish the extent of state transitions when added prior to TDS. These data are consistent with the hypothesis that the redox status of the cytochrome *b_f* low-potential chain plays a role in signaling phycobilisome redistribution during state transitions. Specifically, reduction of the low-potential chain diminishes the dark to light state transition whereas its oxidation allows the state transition.

3.2.2 – The 77K Fluorescence Studies of State Transitions and Phycobilisome distribution.

3.2.2.1 – 77K fluorescence studies provide further insight into regulation of state transitions. Room temperature fluorescence investigations of redox-signaling of state transitions in *Synechocystis* 6803, performed with the BioRad FX scanner, were followed by analysis of 77K fluorescence emission spectra. 77K fluorescence experiments are widely used to evaluate state transitions. Room-temperature and 77K emission spectra from *Synechocystis* 6803 look significantly different in the wavelength range of 600 to 800 nm. At room temperature, one uneven peak is observed; but at 77K, three distinct peaks emerge. Phycocyanin (PC) fluoresces on the far left of the range (650 nm) and arises from the rods or discs of phycobilisomes. PC fluorescence is expected to vary the least under different redox conditions, relative to other peaks, because it has a lower level of fluorescence and great quantities of phycobilisomes smooth out variances in PC changes. Thus, spectra were normalized to the peak corresponding to phycocyanin (PC) fluorescence (650 nm) to compare the relative changes in intensity of PS II (690 nm) and PS I (725 nm) fluorescence between cultures frozen during illumination and in darkness. Allophycocyanin (APC) fluorescence (at 665 nm) was also considered in the data interpretation because changes in this much more variable phycobilisome peak are indicative of detachment (increase) or attachment (decrease) of phycobilisomes (80). The rationale being that allophycocyanin fluoresces more intensely than phycocyanin and is also the core of the phycobilisome complex through which exciton (energy) transfer to the reaction centers is proposed to occur. Thus once the phycobilisomes are detached, one

can think of them as being “unplugged,” and upon excitation with light, exciton energy is lost as fluorescence rather than being transferred to a reaction center. Together these peaks provide information on where harvested-light energy is being shunted (to PS I or PSII), and the allophycocyanin peak can provide information on whether the attachment or detachment of phycobilisomes is a prime contributor to changes in PS I and PS II ratios.

In room temperature studies, we had noted that NQNO impaired the characteristic rise in PS II fluorescence after exposure to light. However, NQNO only impaired this manifestation of the state transition when the inhibitor was added to cells in the dark prior to exposure to light. 77K fluorescence spectrophotometry allows us to probe the question more deeply. The experiments described below investigated the impacts of NQNO and TDS added to cultures in the light versus dark. In all trials, some culture samples were exposed to light and others were kept in the dark during freezing in liquid N₂.

3.2.2.2 – 77K fluorescence spectra of *Synechocystis* cultures treated in the dark and frozen in the dark. In these experiments, cultures in late exponential phase (~1.2 OD₇₅₀) were incubated in the dark for 15 minutes prior to the addition of either TDS (10 μM), NQNO (1 μM), or TDS and NQNO, and freezing in liquid nitrogen (77°K). When spectra from cells frozen in the dark are compared in the emission region of PS I (Figure 12), we see that PS I fluorescence (720 nm) was higher in the control cells compared to cells treated with TDS and/or NQNO. This immediately tells us that both inhibitors promote state-1-type, phycobilisome arrangements (i.e. lower PS I fluorescence normally associated with PQ pool oxidation). These findings suggest that this response does not

arise from a redox-sensing mechanism located between PS II and the cytochrome *bf* complex, that would be expected to increase PS I fluorescence. The lowering of PS I fluorescence by cytochrome *bf* complex inhibitors in the dark suggests two attributes of the redox-sensing mechanism controlling phycobilisome association in the dark. The first is that blocking the cytochrome *bf* complex at the Q_p site with TDS, or the Q_n site with NQNO, had the same effect on a redox-sensing mechanism that functions in the dark. Namely, both decreased fluorescence from PS I. This leads to the second attribute. When the cytochrome *bf* complex was blocked by either TDS or NQNO, PS I fluorescence decreased, indicating that the redox-sensing mechanism must perceive an oxidation event. Conceptually, when electron flow through the cytochrome *bf* complex is blocked, only components downstream of the cytochrome *bf* complex would be oxidized. Thus, these data (Figure 12) allow me to propose that the redox-sensing mechanism that functions in the absence of light to control phycobilisome distribution is primarily independent of the cytochrome *bf* complex. I draw this conclusion because TDS and/or NQNO had the same effect, the effect is inconsistent with PQ pool reduction as the signaling event, and thus the mechanism must sense the redox-state of electron carriers somewhere downstream of the cytochrome *bf* complex.

Relating these 77K data in Figure 12 to room temperature fluorescence data (Figure 10), we see that when inhibitors were initially added to cultures in the dark, room-temperature fluorescence from PS II was also lower than when the inhibitors were added in the light. Recall that PS II fluorescence measured at room temperature includes fluorescence from phycobilisomes and PS I, thus emission from all of these components

is combined. Viewing the more specific fluorescence peaks at 77°K, we see that the lower fluorescence observed at room temperature, when inhibitors were added in the dark, was likely not due to decreased fluorescence from PSII, but more probably to decreased fluorescence from PS I.

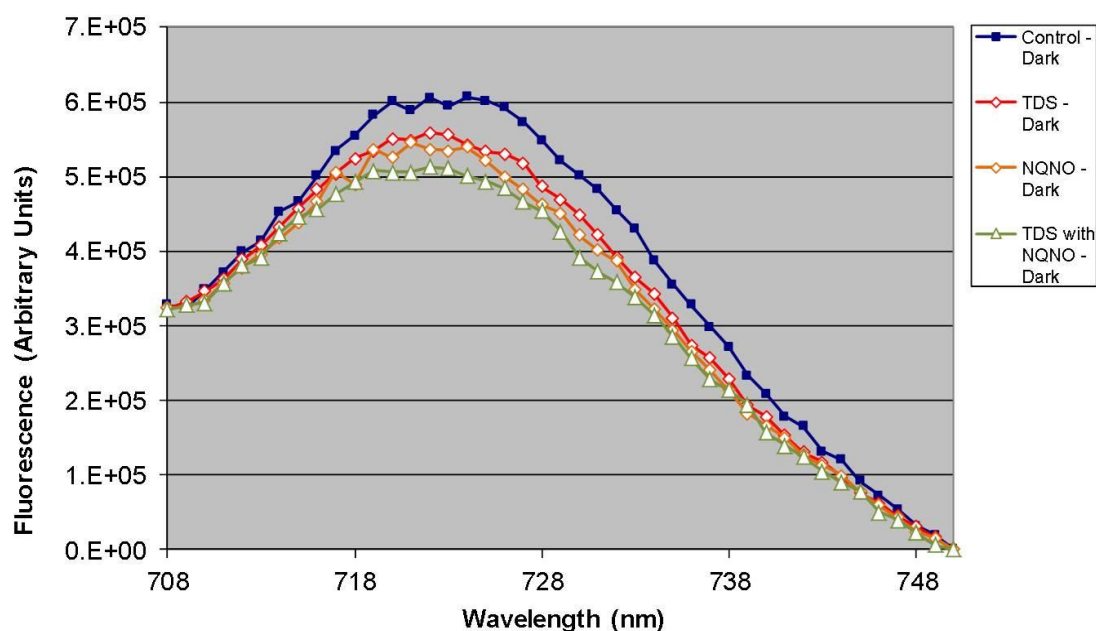


Figure. 12 – 77K fluorescence spectra of PS I peak (720 nm) of *Synechocystis* cells frozen in the dark after treatment in the dark and normalized to PC (650 nm). Cells (~7 mg/ml Chl / 1.1 OD₇₅₀) were incubated in the dark for 15 minutes (Blue) prior to addition of the inhibitors TDS (10 μ M - Red), NQNO (1 μ M - Orange), and TDS with NQNO (Green). The cells were placed within a liquid nitrogen filled dewar in the light-path of a 580 nm excitation light from a xenon arc-lamp. Samples were taken across the spectrum at 1 nm intervals.

In models of phycobilisome-mediated redistribution of excitation energy, fluorescence changes between PS I and PS II are assumed to occur because light energy collected by phycobilisomes is distributed preferentially to either PS I or PS II in response to the redox state of the electron transport chain. Thus, if there is a decrease in PS I fluorescence (Figure 12) we would expect this change to be accompanied by an increase

in PS II fluorescence. In fact, when we look in the range of PS II fluorescence (Figure 13) in the same 77°K fluorescence spectra shown in the previous figure (Figure 12), a small increase in PS II fluorescence in dark-frozen cells was seen only in the TDS-treated samples (Figure 13). This indicates that the redistribution of excitation energy from phycobilisomes functioned as expected in the presence of TDS. Thus, TDS did not appear to impair state-transitions in the dark, which is largely consistent with previous room temperature experiments (Figure 10). NQNO addition, like TDS, did decrease PS I fluorescence (Figure 12); but in contrast to TDS, no accompanying increase in PS II fluorescence was detected (Figure 13). Even when TDS was added together with NQNO, the expected increase in PS II was not observed. Thus, these data suggest two conclusions about phycobilisome redistribution that occurs in the absence of light. If we assume that a decrease in PS I fluorescence would normally be accompanied by redistribution of light energy to PSII, then TDS does not impair the redistribution of phycobilisomes, but does trigger a redox-sensing mechanism that apparently senses oxidation in the electron transport chain downstream of the cytochrome *bf* complex. NQNO also causes a redox-sensing mechanism to perceive a similar oxidation signal, but further appears to interfere with the redistribution of phycobilisomes or light-energy transfer to PS II even in the presence of TDS. This is consistent with the idea that the redistribution of phycobilisomes in the dark to PS II is dependent on either the redox status of the cytochrome *bf* complex low-potential chain or the occupancy of the Q_n site by NQNO.

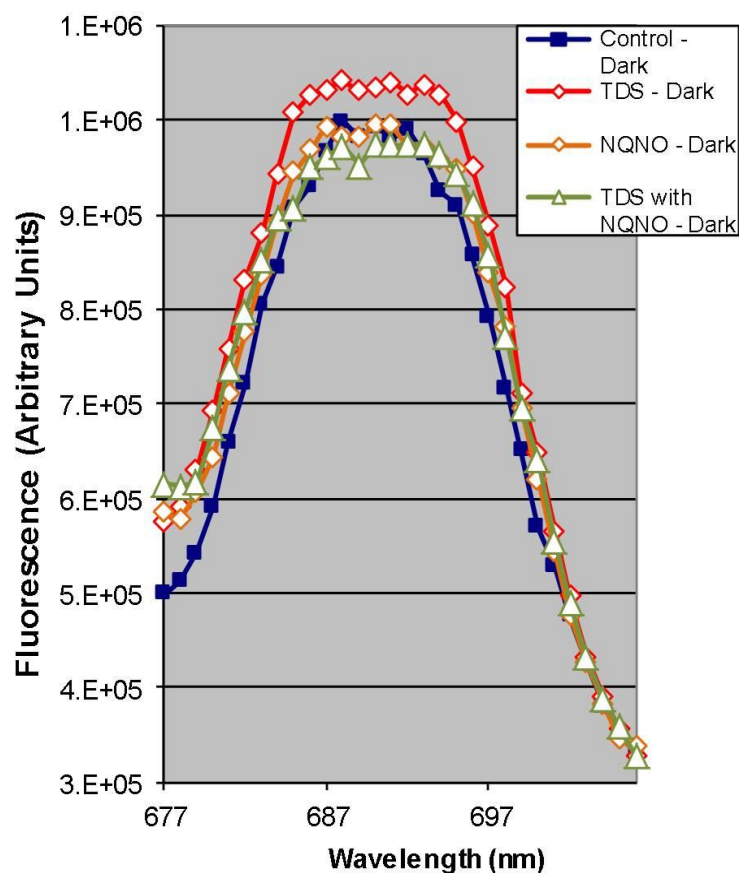


Figure. 13 – 77K fluorescence spectra of PS II peak (690 nm) of *Synechocystis* cells frozen in the dark after treatment in the dark and normalized to PC (650 nm). Cells (~7 mg/ml Chl / 1.1 OD₇₅₀) were incubated in the dark for 15 minutes (Blue) prior to addition of the inhibitors TDS (10 μ M - Red), NQNO (1 μ M - Orange), and TDS with NQNO (Green). The cells were placed within a liquid nitrogen filled dewar in the light-path of a 580 nm excitation light from a xenon arc-lamp. Samples were taken across the spectrum at 1 nm intervals.

3.2.2.3 – 77K fluorescence spectra of *Synechocystis* cultures treated in the dark and frozen in the dark or frozen during illumination. Replicates of the dark incubated and inhibitor-treated samples from the previous experiment (Figures 12 and 13) were exposed to light for 5 minutes and then frozen in liquid nitrogen while under illumination (Figure 14). This allows the fluorescence spectra of cultures in the dark to be compared with those in response to light after both samples had been treated in the dark with

inhibitors. In these spectra, the redox-specific effects of TDS, NQNO, and TDS together with NQNO are apparent.

Untreated, control cultures responded as expected (Figure 14 - Panel A). PS I fluorescence decreased and PS II fluorescence increased as the unobstructed electron transport chain moved from a reduced state (State 2) in the dark to an oxidized state in the light (State 1). Thus as this state transition occurs, we assume normal movement of phycobilisomes (PBS) and normal energy transfer, allowing the PBS from PS I to distribute more of their energy to PSII. In contrast, NQNO-treated cells (with NQNO added in the dark) showed little or no PS II fluorescence increase when exposed to light (Figure 14 - Panel C), demonstrating that state transitions were inhibited. These data confirm the results of the room temperature fluorescence studies presented in Figure 10. TDS treatment (with TDS added in the dark) did not greatly impair the state-transition (Figure 14 - Panel B), in agreement with previous room temperature experiments (Figure 10). In the sample treated with TDS and NQNO (with inhibitors added in the dark), the increase in PS II fluorescence, and thus the state transition, was not greatly impaired despite the presence of NQNO (Figure 14 - Panel D). This finding is in agreement with the room temperature studies that were performed (Figure.11).

Replicates of the 77K experiments (with NQNO and TDS) showed agreement with room temperature experiments (Figures 10 and 11) when the exposure time to the inhibitor before freezing the sample was increased. These findings suggest that NQNO, when added to cultures in the dark, impairs the state transition by either reduction of the low potential chain in the cytochrome *bf* complex or by physically occupying the Q_n site.

However, the fact that TDS applied together with NQNO appears to rescue NQNO-impairment of the state transition suggests that TDS binding changes the Q_n site in such a way as to lessen the impact of NQNO. Room temperature and replicate 77K experiments mentioned above indicate that this may be a dynamic process and the longer the organism is exposed to the inhibitors NQNO and TDS, the more pronounced the NQNO-specific impairment of the state transition. Thus it appears that NQNO has an immediate impact on the redistribution of PBS energy that can be immediately countered by the presence of TDS, but over time TDS may become less able to inhibit the effect of NQNO until some equilibrium is reached. If this balancing occurs it would suggest that TDS and NQNO must have opposing effects on the component of the redox sensing/signaling mechanism of the state transition. One component fitting this description would be the *b*-hemes, or perhaps heme c_i (c_n) of the cytochrome *bf* complex.

One final observation from these experiments is that PS I fluorescence in all the treated samples did not decrease in response to light as it did in the control untreated cells (Figure 14). If we were to compare the level of PS I fluorescence among all the samples in response to light (done but not shown), we would see that they had the same level. This means that, relative to PS I fluorescence, all of the treated cultures were already in a state 1 condition, normally expected if the plastoquinone pool were oxidized. This is in agreement with the conclusions drawn from Figure 12.

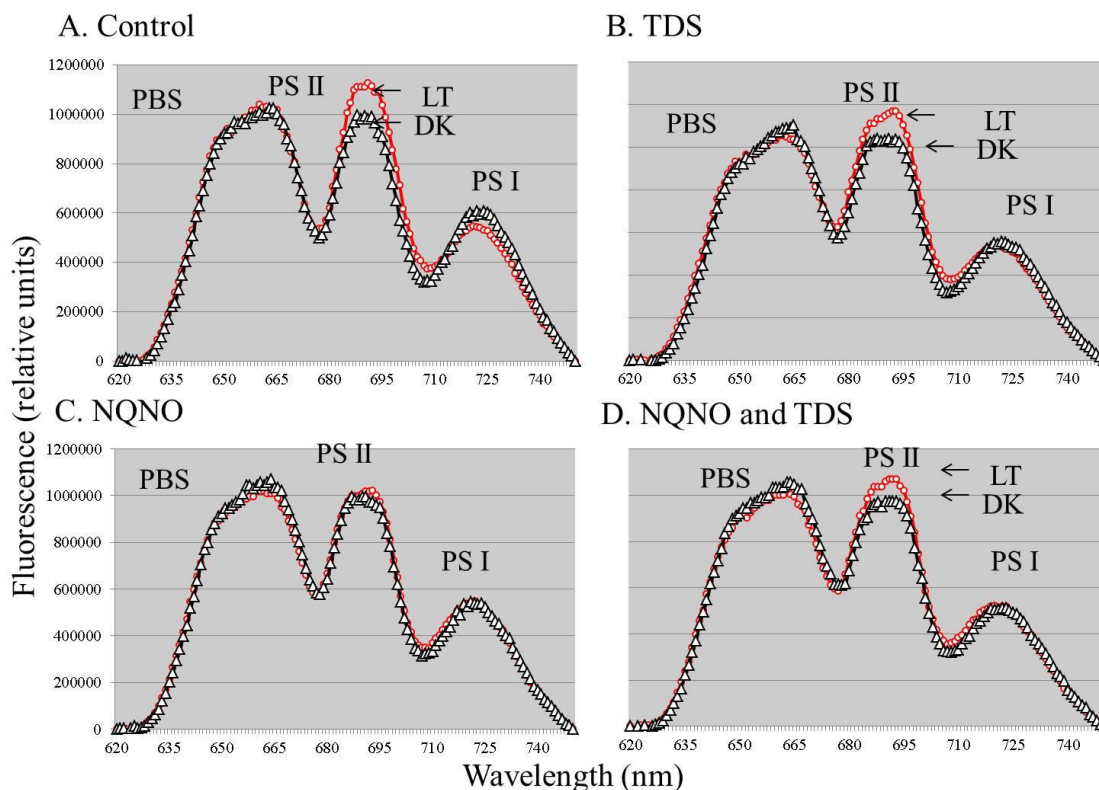


Figure. 14 – 77K fluorescence spectra of *Synechocystis* cells frozen in the light or dark after treatment in the dark. Cells (~ 7 mg/ml Chl / 1.1 OD_{750}) were incubated in the dark for 15 minutes prior to addition of the inhibitors TDS (10 μ M) and/or NQNO (1 μ M). The cells were then frozen in the dark (Black Triangles) or after exposure to light (Red Circles) ($50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and placed within a liquid nitrogen filled dewar in the light-path of a 580 nm excitation light from a xenon arc-lamp. Samples were taken across the spectrum at 1 nm intervals. The fluorescence spectra have been normalized to PC at 650 nm.

3.2.2.4 – 77K fluorescence spectra of *Synechocystis* cultures treated with inhibitors during illumination. The following sections describe experiments in which cells were treated with the same regimen of inhibitors as above (Figure 14). However, the inhibitors were added to cells under ambient room light ($\sim 50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) prior to 15 minutes of dark incubation, followed either by freezing in liquid nitrogen (77K) in the dark or after 5 minutes of incubation in the light (Figure 15). The resulting 77K spectra were then normalized to the phycocyanin (PC) emission peak at 650 nm.

One identifiable difference in the light-treated samples (Figure 15) is the asymmetry of the phycobilisome-allophycocyanin peak (665 nm) relative to the phycobilisome-phycoerythrin peak (650 nm) in samples treated with NQNO or treated together with NQNO and TDS. This suggests that upon addition of NQNO to cultures in ambient room-light, a significant association of phycobilisomes occurs with the photosystems because the allophycocyanin fluorescence emission (665nm) decreases, indicating attachment of PBS to PS II or PS I (Figure 15, Panel C). Initially this seems counterintuitive because there is no appreciable difference in fluorescence from PS II in the cells frozen in the light or dark after treatment with NQNO. However, when we compare the spectra from cells frozen in the dark, the level of PS II (~695 nm) relative to PC fluorescence at 650 nm, in NQNO-treated samples (Figure 15, Panels C and D) was higher than in either the control or samples treated with TDS alone (Figure 15, Panels A and B, respectively). This indicates that when NQNO is added, this causes additional phycobilisome harvested, light-energy to be shunted to PSII. When replicate samples were frozen in the light, the sample treated with NQNO alone did not show appreciably increased PS II fluorescence relative to dark-frozen samples (Figure 15 Panel C). However, the sample that included both NQNO and TDS did show a PS II fluorescence increase (Figure 15, Panel D). Additionally we see that the marked change in APC to PC fluorescence in the NQNO-treated sample (Figure 15, Panel C) appears diminished, at least in the sample treated with TDS alone (Figure 15, Panel B). This suggests that the original effect of NQNO (the shunting of some harvested light energy to PSII) might be diminished in the presence of TDS. Indeed, the PS II (695 nm) peak relative to PC

emission (655 nm) from dark-adapted cells treated with TDS and NQNO (Figure 15, Panel D, black PS II peak at 695 nm) is somewhat lower relative to that from dark-adapted cells treated with NQNO alone (Figure 15 Panel C, black PS II peak at 695 nm). However, this distinction is not clear from these data. The tentative conclusion that can be drawn from the spectra from light-adapted cells is that TDS may counter, to some extent, the NQNO-mediated attachment of phycobilisomes as indicated by the smaller ratio drop in the APC emission peak (665 nm) in cells treated with TDS and NQNO (Figure 15, Panel D) compared to cells treated with NQNO alone (Figure 15, Panel C)

As reasoned above, differential effects and their mitigation by competitive additions of TDS and NQNO indicate differences in the asymmetric, 650-665 nm phycobilisome fluorescence emission peaks. These data suggest that increased attachment of PBS to PS II in the NQNO-treated samples is an event mediated by the redox state of the cytochrome *bf* complex low-potential chain. Overall, the principal conclusions from both the room-temperature (Figures 10 and 11) and 77K fluorescence (Figures 12-15) studies are that NQNO, an inhibitor of the cytochrome *bf* quinone-reductase (Q_n) site and low-potential chain, inhibits redox-signaling of state transitions when added to cells in the dark but not when added to cells in the light.

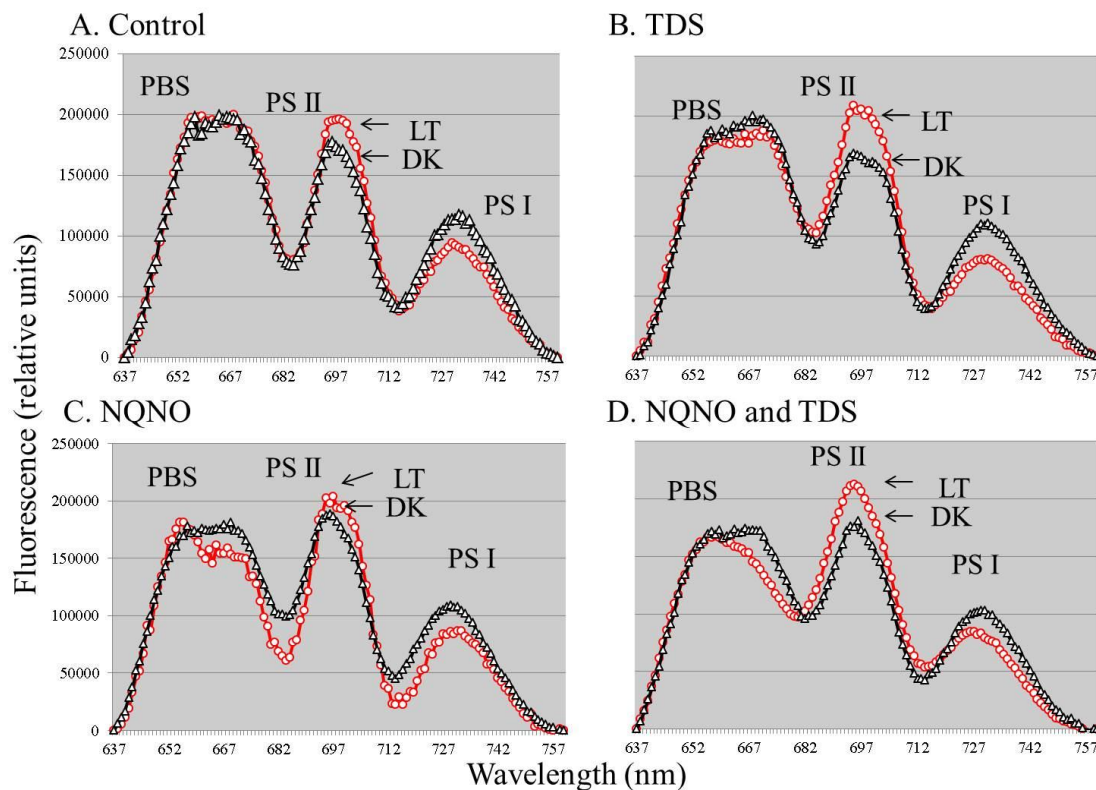


Figure. 15 – 77K fluorescence spectra of *Synechocystis* cells frozen in the light or dark after treatment in the light. Cells (~ 1.4 OD₇₅₀) were treated with TDS (10 μ M) and/or NQNO (1 μ M) in ambient room light prior to incubation in the dark for 15 minutes. Cells were then frozen in the dark (Black Triangles) or after exposure to light (Red Circles) (50 μ mol photons m⁻² s⁻¹) and placed within a liquid nitrogen filled dewer in the light-path of a 580 nm excitation light from a xenon arc-lamp. Samples were taken across the spectrum at 1 nm intervals. The fluorescence spectra have been normalized to PC at 650 nm.

Chapter 4: Discussion

4.1 – Sources of Reactive Oxygen Species Production

4.1.1 - Reactive Oxygen Species Production in *Synechococcus* PCC 7002.

4.1.1.1 – ROS production in the wild type compared to the cytochrome *bf* low- and high-potential chain mutants. It has previously been shown that superoxide production in the wild type is exacerbated in the light by inorganic carbon limitation or in the dark in cells supplemented with glycerol (27). These conclusions are consistent with the idea that ROS increases when the electron transport chain is backed-up. Lowering CO₂ concentration to ambient levels (0.035%) from 3% CO₂ removes the electron acceptor for the Calvin Cycle and thus removes a significant sink for NADPH oxidation. Thus NADPH builds up, and the terminal acceptor for linear electron transport, NADP⁺, becomes limiting. The result is that electrons flowing into PS I have nowhere to go, the cytochrome *c*₆ pool becomes reduced, and thus turnover of the cytochrome *bf* complex slows because oxidized cytochrome *c*₆ is not available to be reduced. In the dark, in cells supplemented with glycerol, the respiratory electron transport chain uses the cytochrome *bf* complex to reduce cytochrome *c*₆, which is oxidized by cytochrome oxidase. Thus the higher level of superoxide observed in the dark likely arises from electron transfer to O₂ from the cytochrome *bf* complex or other respiratory electron transport components (27).

During dark incubation in the presence of glycerol, the cytochrome *bf* high-potential chain, PetC1-Δ2G mutant did not produce significantly more superoxide than the wild type (27). However, the low-potential chain mutant PetB-R214H mutant

produced 25 times more superoxide than the wild type under these conditions. Overall rates of electron flow through the cytochrome *bf* complex were similar in the PetC1-Δ2G and PetB-R214H mutants. This was measured with both whole-chain electron transfer (O_2 evolution in intact cells with bicarbonate as the electron acceptor, ref. 27), and as cytochrome *f/c*₆ re-reduction rates in intact cells (following a short illumination of a few ms). These rates were at least four times slower in both mutants ($t_{1/2} \sim 40$ ms) than in the wild type ($t_{1/2}$ 5-9 ms, ref. 34, and unpublished). Thus the plastoquinone pool should be comparably reduced in both mutants and this argues against the hypothesis that increased superoxide production in the PetB-R214H mutant results from increased “back-pressure” (i.e. electrons “piling up”) on elements upstream of the cytochrome *bf* complex. An alternative hypothesis is that increased superoxide production in the PetB-R214H mutant results from the slower turnover of the cytochrome *bf* low-potential chain and slower oxidation (longer half-life) of the reactive semiquinone produced after the initial oxidation of plastoquinol at the cytochrome *bf* Q_p site.

Data presented here (Figure 5) demonstrate that increased production of superoxide of the PetB-R214H mutant may be primarily responsible for the slower growth-rate (increased doubling time) of this mutant. These experiments show that anoxic conditions decreased the doubling time of the PetB-R214H mutant (i.e. increased the growth rate); whereas the growth rates of the wild type and PetC1-Δ2G mutant were unaffected by this treatment. However, the increased production of superoxide in PetB-R214H was probably not the only growth-limiting factor in this mutant. Even after removing the O_2 substrate for superoxide production, by gassing with N_2/CO_2 , the

doubling time of PetB-R214H was still significantly longer than that of the wild type or PetC1-Δ2G mutant (Figure 5).

Since DCMU was not added to prevent O₂–evolution from photosystem II in these experiments, some superoxide could still be produced to account for the remaining inhibition of growth in the PetB-R214H mutant. This could be tested by growth experiments under heterotrophic conditions (cells incubated under illumination with DCMU and N₂/CO₂ gas flow). The PetB-R214H mutation in the cytochrome *bf* low-potential chain might also disrupt redox signaling of light-harvesting phycobilisome distribution or cyclic electron flow around photosystem I, and thereby limit photosynthetic efficiency and growth.

4.1.1.2 – Probing ROS production with H₂DCFDA. The highly fluorescent ROS probe H₂DCFDA (68) readily diffuses into cells where it is cleaved by endogenous esterases into the ROS-sensitive H₂DCF. In this form, the probe is particularly (though not exclusively) sensitive to the hydroxyl radical, a common break-down product of hydrogen peroxide and a good indicator of this highly toxic form of ROS. Superoxide is readily transformed by superoxide dismutase into hydrogen peroxide which can be rapidly catalyzed via the Fenton Reaction to yield hydroxyl radicals (75). Once ROS encounters H₂DCF, protons (as H₂) are lost forming DCF which fluoresces brightly at ~530 nm. Initial assays for ROS production with H₂DCFDA were performed in *Synechococcus* 7002 PetB-R214H and the wild type (Figure 6). Based on DCF fluorescence, PetB-R214H produced significantly more ROS than wild type cells incubated in the dark in the presence of glycerol. These data confirm Darryl Horn's

findings with the optical superoxide probe XTT (27) (Materials and Methods, Section 2.5), and provide evidence for rapid conversion of superoxide into hydrogen peroxide and other downstream oxygen radicals. As indicated in the literature (76) and confirmed through experimentation (data not shown), available iron in the assay media had to be removed to eliminate background fluorescence from the DCF ROS probe. Thus all experiments with H₂DCFDA were performed in the absence of iron as described in Materials and Methods. Removal of iron also induces apparent ROS production in cyanobacteria (77). However, fluorescence from iron removal was a relatively small component of the DCF signal and assumed to be part of the background signal in these experiments.

4.1.1.3 – Does the quinone-reductase site (Q_n) inhibitor NQNO mimic the effects of the *PetB-R214H* mutation? The inhibitor, NQNO (2-n-nonyl-4-hydroxyquinoline N-oxide), inhibits the Q_n site of the cytochrome *bf* complex in isolated chloroplast thylakoid membranes at concentrations of $\sim 1 \mu\text{M}$ (71, 72) and thereby increases reduction and delays oxidation of the *b*-hemes (34, 85). At higher concentrations, NQNO also binds the Q_p site of cytochrome *bc* complex (86). The *PetB-R214H* mutation similarly inhibits *b*-heme oxidation and increases their reduction level (34). Thus NQNO and the *PetB-R214H* mutation appear to have similar effects on the cytochrome *bf* low potential chain and quinone-reductase (Q_n) site.

4.1.1.4 – ROS production mediated by NQNO and the *PetB-R214H* mutation.

As in the *PetB-R214H* mutant, treatment with NQNO increased ROS production, based on DCF fluorescence (Figure 7). This was observed both in the wild type and in the *PetB-*

R214H mutant. In these experiments, illumination further increased ROS production relative to dark incubations. In other experiments, ROS was found to be greatest under dark conditions in PetB-R214H, as in previous studies (27). One primary difference in the current study and that of Horn (27) is that his dark incubated *Synechococcus* cells were supplemented with glycerol whereas those in my study with H₂DCFDA were not. Thus the absence of a respiratory chain substrate (glycerol) might explain why the current study found a lower level of ROS during dark incubation compared to the findings by Horn (27). Another possibility is that the DCF probe was auto-oxidized in the light. However, ROS production in the PetB-R214H mutant, measured with H₂DCFDA, was still greater than in the wild type. A final consideration is the relative sensitivities of the XTT and DCF probes to different forms of ROS. XTT, used by Horn (27), is very sensitive and specific for superoxide, whereas H₂DCFDA is more sensitive to hydroxyl radicals, peroxynitrite and hydrochloride anions (76). This would make XTT much more specific to short-lived superoxide locally produced from the electron transport chain, whereas H₂DCFDA might more generally measure the accumulation of ROS from anywhere in the cell. For these reasons, the quantities and forms of ROS produced by the two studies cannot be compared directly. Each has its advantages. The H₂DCFDA detects ROS from many different sources but lacks the specificity of XTT for detecting superoxide. Finally, the experiments performed with the optical XTT probe were lengthy, requiring incubations of several hours. In experiments with H₂DCFDA, strong signals appeared after much shorter periods and prolonged incubations in Fe-starved conditions had to be avoided because they artificially enhanced ROS production (77)

In comparing ROS production rates measured with H₂DCFDA in the light relative to darkness (Figure 7), cells in the light produced more ROS than cells in the dark. Addition of NQNO to cells in the light resulted in a 4-fold increase relative to cells in the dark in the wild type, but only a 2-fold increase in PetB-R214H. Thus we can surmise that NQNO further exacerbated ROS production in the PetB-R214H mutant, suggesting that the sites that induce ROS production, from the mutation and NQNO addition, overlap to some extent. This likely results from the similar impacts of the PetB-R214H mutation and the NQNO inhibitor; namely, both impair oxidation of the cytochrome *bf* low potential chain. If the H₂DCFDA probe is not limiting, then we can assume that in these experiments, ROS produced by PetB-R214H during light incubation in the presence of NQNO may represent the maximal level of ROS production.

Work by Alric et al. (48) shows that NQNO binding to the cytochrome *bf* Q_n site lowers the mid-point potential of the c_n heme, located near the Q_n site, by ~250 mV. This drop in redox potential of the c_n heme from +100mV to -150 mV on binding NQNO, and the proximity of the Q_n site to oxygen in the cytoplasmic compartment, could provide another avenue for superoxide production. This lower-potential c_n heme could be the site of the additional ROS production observed on addition of NQNO to the PetB-R214H mutant. Thus it is conceivable that NQNO, as well as the PetB-R214H mutation, may induce ROS formation from more than one site. Both cause over-reduction of the cytochrome *bf* low-potential chain and thus ROS may be produced from a longer-lived plasto-semiquinone at the plastoquinol-oxidation (Q_p) site. Alternatively, or in addition, NQNO may induce ROS formation at the Q_n-site by lowering the midpoint potential of

heme c_n . The impact of the PetB-R214H mutation on heme c_n is not known, but removal of the positively-charged Arg 214 from the Q_n -pocket may similarly lower the midpoint potential of heme c_n and cause it to become a site of ROS production.

4.1.1.5 – PetB-R214H in the context of the Dual Plastoquinol Oxidation Model.

The cytochrome *bf* complex, Q_p -site has been postulated as a source of ROS production (34, 27) for reasons detailed above (Introduction 1.2.4). Contrary to this proposed mechanism is the model of dual plastoquinol oxidation (29). In this scenario, the Q_p site is cavernous enough to allow two quinol head-groups to fit in beside one another and electrons to pass through the high- and low-potential chains nearly instantaneously. This would leave behind two semiquinones which subsequently dismutate and stabilize to form one plastoquinol molecule and one plastoquinone. The plastoquinone diffuses out to be replaced by another quinol and the process begins again. The Q_p site is large enough to support this hypothesis (35) and there is ongoing discussion as to whether the high and low potential chains must necessarily fire in series (29). Thus simultaneous oxidation of two plastoquinol molecules at the Q_p site is a possibility. Compared to the single plastoquinol-oxidation model described above, one might surmise that if indeed little or no ROS is produced from the Q_p site, then the dual plastoquinol-oxidation model might more accurately describe the source of ROS formation in the cytochrome *bf* complex. In the context of this model, the source of ROS production in the PetB-R214H mutant and upon NQNO addition might occur exclusively at the Q_n site. However, semiquinone may still form in the PetB-R214H mutant operating by the dual-quinol oxidation mechanism. If one quinol is being oxidized (via the Rieske iron-sulfur protein) to a semiquinone and

the other not (because the *b*-hemes are already reduced) then there would be no dismutation of semiquinone and elimination of two semiquinones. Thus ROS production of the PetB-R214H mutant measured via XTT or H₂DCFDA is compatible with either single or dual quinol oxidation schemes.

4.1.2 – Reactive Oxygen Species production in *Synechocystis* PCC 6803.

4.1.2.1 – ROS in *Synechocystis* compared to *Synechococcus*. No direct comparisons were made between *Synechocystis* 6803 and *Synechococcus* 7002. *Synechocystis* 6803 expresses quinol oxidase, a membrane spanning protein which can catalyze the oxidation of plastoquinol directly from the plastoquinone pool (26).

4.1.2.2 – ROS production in the context of putative ROS sources. NQNO exacerbates ROS production in *Synechocystis* 6803 (Figure 8) as it did in *Synechococcus* 7002 (Figure 7). I assume that the mechanism of the plastoquinol oxidation at the cytochrome *bf* complex in *Synechococcus* 7002 and *Synechocystis* 6803 is not vastly different. TDS blocks at the Q_p site, thus it likely inhibits ROS production by sterically hindering oxidation of plastoquinol. However, TDS would also inhibit ROS production at the Q_n site by limiting electron flow to O₂ via the Q_n site. NQNO should still increase ROS production at the Q_p site if semiquinones could be formed and also at the Q_n site by lowering the midpoint potential of the *c_n* heme. Limiting plastoquinonol oxidation at the Q_p site would limit ROS production in either case. Thus it is expected that TDS should lower ROS production in either case, which it did.

4.1.2.3 – ROS production in the presence of TDS and/or NQNO. Figure 11 shows that [NQNO] at 250 nM or 5 μ M increased ROS in culture samples of *Synechocystis* 6803 ($OD_{750} = 0.41$). This figure also show that [TDS] at 1 or 10 μ M decreased ROS in samples from this same culture. Furthermore, the experiment shows that the impacts of NQNO at 250 nM or 5 μ M is counteracted by 1 and 10 μ M concentrations of TDS. However, the level of ROS production in these samples did not drop to control levels. This suggests that the residual ROS production arose from a low flux of electrons that are still passing through the low potential chains. With NQNO present to block oxidation of semiquinones at the Q_n site, and lower the midpoint potential of the c_n heme, ROS production could still occur by the mechanism described in section 4.1.1.5.

4.1.2.4 - Conclusions of ROS production studies in *Synechocystis* PCC 6803.

Data presented here (Figure 11) suggest that the primary source of ROS production from the cytochrome *bf* complex could be either the Q_p site or the Q_n site. TDS lowered ROS production whether NQNO is present or not. But, TDS also reduced the flow of electrons through the low potential chain to the Q_n site. The muddling factor in all this appears to be the NQNO-specific effect on the Q_n site. Thus it becomes impossible to say at which site the ROS production originates. What can be said is that blocking quinone reduction at the Q_n site increases ROS production and quite possibly in two different ways.

4.2 – Mechanisms of State Transitions

4.2.1 – Phycobilisome-mediated PS II fluorescence, state transitions, and the plastoquinone pool redox status.

During photosynthetic electron transport there may be more energy available to the photosystems than can be used. Excess energy can be dangerous as prolonged excited states of chlorophyll can increase the incidence of energy passed to O₂ forming singlet oxygen (see Introduction section 1.1.1). Often excess energy is lost via conversion to fluorescence from chlorophyll and phycobilisomes, which are the primary fluorescent molecules during photosynthesis. If more light energy is funneled to the photosystem, then the energy lost via fluorescence should also increase. Chlorophylls bound within protein complexes, such as those found in the photosystems, fluorescence at different wavelengths than those bound to the peripheral antenna because the protein environment affects the energy emitted as fluorescence. Subtle changes in wavelength have been well characterized, showing PS I fluorescing maximally around 720 nm and PS II around 680 nm. Thus the relative fluorescence changes of 680 nm and 720 nm provide information on the direction of electron transport toward oxidation (720 nm fluorescence increase) or reduction (680 nm fluorescence increase) of the PQ pool. Generally, PS II fluorescence is more intense than PS I fluorescence; however, changes in the metabolic environment can change photosystem stoichiometry via transcriptional regulation or induce changes in the arrangement of the photosynthetic complexes that alter fluorescence. One example is the formation of the IsiA antenna system around PS I trimers in response to Fe-starvation (78). Under these conditions PS I may contribute significantly to photosystem

fluorescence due to a much larger optical cross-section. Regardless of the initial fluorescence distribution, changes in light intensity, quality, or changes in the redox state of the electron transport chain, cause immediate shifts of the PSII:PSI ratio. Such changes were observed using both room temperature fluorescence imaging and 77K fluorescence emission spectrometry (Materials and Methods Sections 2.4 and 2.6, respectively). These adaptive shifts in cyanobacterial light-harvesting complexes are the result of preferential transient association of phycobilisomes with either PS I or PSII. Phycobilisome association increases the optical cross-section of a photosystem and thus increases the harvested light funneled to that reaction center. This also increases the fluorescence from the photosystem, the component that is measured. Chlorophyll excitation with ~480 nm light is not highly absorbed by phycobilisomes, and thus the energy would not funnel to the reaction center through phycobilisomes. Excitation at ~ 532-580 nm preferentially excites phycobilisomes (15), thus phycobilisome-harvested light energy will funnel to the photosystem with which the phycobilisomes are associated. The subsequent photosystem fluorescence is a measure of the phycobilisome-mediated photosystem fluorescence. In the room temperature fluorescence experiments an emission filter is used to block all light except a narrow bandwidth of 680 nm +/- 5 nm. Thus in response to excitation with a 532 nm laser we record the image of PS II (680 nm) fluorescence, which is a measure of phycobilisome-mediated PS II fluorescence. Changes in phycobilisome-mediated PS II fluorescence represent the changing association of phycobilisomes with PSII, which is dependent upon the redox state of the electron transport chain. Thus phycobilisome-mediated PS II fluorescence is an indirect measurement of the redox state of the

plastoquinone pool. Under reduced conditions the electron transport chain signals phycobilisomes to associate with PS I and as the plastoquinone pool becomes more oxidized more phycobilisomes associate with PSII. These redox-dependent changes in phycobilisome-mediated PS II fluorescence are a measure of the state transition.

The mechanisms by which these changes in light intensity or quality are sensed have been the source of much debate. It is unclear what components of the electron transport chain sense and/or signal the transient association of phycobilisomes as there have been conflicting reports (61, 18). The work presented here suggests that the cytochrome *bf* complex is one of two redox-sensitive mechanisms mediating the transient redistribution of phycobilisomes between PS I and PS II reaction centers. Most of the key findings of the room temperature fluorescence imaging studies (Figures 10 and 11) have been further corroborated by fluorescence emission spectroscopy of cultures frozen at 77K (Figures 12-15) and in room temperature fluorescence kinetics experiments with a Biologic JTS-10, Joliot-type spectrophotometer to observe light-induced variable fluorescence (data not shown). These combined data suggest that two different redox sensing/signaling mechanisms function together to redistribute phycobilisomes, one exclusively operating in the presence of light.

4.2.2 – State Transitions Occur Despite Inhibition of Plastoquinone Pool Oxidation.

TDS inhibits oxidation of the plastoquinone pool via the cytochrome *bf* complex. Thus, the plastoquinone pool becomes more reduced and, by phycobilisome-mediated PS II fluorescence, a corresponding reduction in PS II fluorescence is observed in room

temperature studies (Figure 10). NQNO produces a similar effect on PS II fluorescence in room temperature studies, possibly because it inhibits a quinol oxidase. Phycobilisome-mediated PS II fluorescence traces of NQNO and TDS treated *Synechocystis* 6803 cultures showed dramatic increases in PS II fluorescence upon exposure of the cultures to light (Figure 10). These state transitions were comparable to the control, although the signal from TDS-treated cultures was often higher. This greater change in PS II fluorescence in TDS-treated samples may be due to a lower PS II fluorescence prior to illumination. Evidence for this is that traces normalized to control time-points show that the control and TDS-treated cultures reached similar levels of PS II fluorescence (Figure 10). During illumination of TDS-treated cultures, we can imagine that the plastoquinone pool is reduced because TDS blocks its oxidation via the cytochrome *bf* complex. Despite this reduction of the plastoquinone pool, we observe PS II fluorescence increasing as if PQ pool oxidation is being sensed by the redox sensing mechanism. Thus phycobilisome redistribution in the light has been uncoupled from the standard model of redox sensing, the plastoquinone pool. It is possible that quinol oxidases or alternative respiratory terminal oxidases may oxidize the plastoquinone in place of the cytochrome *bf* complex. This hypothesis was tested by adding a high (200 μ M) concentration of KCN to inhibit all known respiratory oxidases of cyanobacteria (33). State transitions were still present under these conditions (data not shown) implying that the cytochrome *bf* complex or some downstream mechanism may play an important role in sensing electron transfer chain oxidation, and in signaling state transitions.

Another implication of these observations is that the mechanism for sensing and signaling state transitions may have separate light and dark components. Prior to illumination an unbalanced redox state of the electron transport chain (reduced upstream of the cytochrome *bf* complex and oxidized downstream) likely existed. This suggests that the state transition only operated to transfer phycobilisomes to PS II in response to oxidation when light was present. In the dark, phycobilisomes change their associations between PS I and PS II in response to changes in the electron transport chain redox status. Thus, it cannot be concluded that the state transition mechanism only functions in the light. Yet, there appears to be a conflict with the traditional model of sensing/signaling state transitions because one would have expected the signaling of phycobilisome redistribution to have remained consistent between light and dark environments. Thus, there appear to be two separate mechanisms for sensing and signaling state transitions. Illumination seems to determine which is more active or whether the same mechanism operates with changing light/dark sensing/signaling domains (meaning chemical/enzymatic attributes that distinguish stimuli. These findings are consistent with the implication that the cytochrome *bf* complex low-potential domain functions in a redox sensing mechanism in response to light. The cytochrome *bf* complex might also then be a site for signaling state transitions, requiring components which directly or indirectly interact with phycobilisomes or a component thereof.

4.2.3 – Treatment with NQNO in the Dark Significantly Inhibits the State Transition.

If NQNO is added to cultures of *Synechocystis* 6803 in the dark, the state transition is significantly inhibited (Figure 10). TDS does not show the same effect upon addition in the dark. NQNO increases reduction of the *b*-hemes when added in the dark or under illumination (data not shown). Thus the cytochrome *bf* complex low potential chain and plastoquinone pool are more reduced in the presence of NQNO, but the effect on state transitions is not present when NQNO is added in the light. Thus, it seems that the redox state of the low potential domain may determine phycobilisome redistribution specifically in response to light. This conclusion comes from the fact that TDS produces an imbalanced electron transfer chain (see above section 4.2.1.2) in which the low potential chain of the cytochrome *bf* complex is oxidized. This occurs because TDS occupies the Q_p site and prevents electron flow into the the high and low potential chains. If NQNO impairs turnover of the cytochrome *bf* complex (see above section 4.2.1.1), then this inhibitor also produces an imbalanced electron transport chain, except that the low potential chain is reduced in the presence of NQNO. This fundamental difference appears to determine inhibition of state transitions. Thus the new hypothesis arising from data presented here is that the redox status of the cytochrome *bf* low potential chain relays the redox status of the electron transport chain in cells during illumination and is involved in signaling state transitions.

4.2.4 – Sequential Additions of TDS and NQNO to Identify Redox Properties of the Cytochrome *bf* Q_n Site that may Mediate State Transitions.

The hypothesis being tested is that the redox status of the cytochrome *bf* low-potential domain determines the efficiency or effectiveness of binding events at the Q_n site of the complex. To determine whether NQNO binding or the redox state of the low potential chain inhibit state transition, TDS and NQNO were added to the samples in the dark with either TDS or NQNO added first (Figure 11). Assumptions were that 1) the cytochrome *bf* complex low potential domain is reduced in the dark, 2) when NQNO is added the low potential domain remains reduced, and 3) NQNO occupies the Q_n site of the cytochrome *bf* complex. If TDS is added in the dark, instead of NQNO, the low potential chain should become oxidized. Another assumption is that if TDS is added first to oxidize the low potential domain, and then NQNO is added, the low-potential domain should remain oxidized. This should create a conditions where NQNO is bound to the Q_n site but the low potential domain remains oxidized. Indeed, treatment with TDS before NQNO addition appeared to be less effective at inhibiting the state transition than adding NQNO first and then TDS (Figure 11). These results imply that both the low-potential domain redox status and binding events may determine signaling of state transitions. Additionally these results suggest that the redox status of the low potential domain or binding at the Q_p site determine the effectiveness of binding events at the Q_n site of the cytochrome *bf* complex. These results cannot tease apart what effect which binding location has on the other, but they do show that there is a polarity to this relationship. NQNO effects at the Q_n site more strongly regulate state transitions than events at the Q_p

site, because TDS addition after NQNO addition could not restore the state transition, whereas NQNO added after TDS still inhibited state transitions. Others have suggested that binding events at the Q_n site may affect binding at the Q_p site (79). My hypothesis may be consistent with these proposals. My findings indicate that the two quinone binding sites of the cytochrome *bf* complex are certainly linked and function antagonistically.

If we assume that the low potential chain is more reduced in the dark than in the light, then one can infer that the redox state of the low potential chain is sensitive to illumination. Treatment of *Synechocystis* cells with TDS did not appear to be redox-sensitive because the same effects were observed when added in the dark or the light (Figure 10). The impact of NQNO did appear to be redox sensitive. The *b*-heme redox kinetics were found to be sensitive to pH, showing that as the concentration of FCCP (a protonophore that collapses the pH gradient across the membrane) increased, *b*-heme oxidation became inhibited (41).

The midpoint potential of the c_n heme of the cytochrome *bf* complex has been found to range in value dependent on surrounding pH from about +160mV at pH 6 to +20mV at pH 9 (48). This suggests that changes in pH may also play a role in modulating state transition signaling by the cytochrome *bf* complex. As Barbagallo and colleagues (41) showed, raising the pH inhibits *b*-heme oxidation. This is consistent with Alric et al. (48) finding that higher pH lowers the midpoint potential of the c_n heme, which should result in greater reduction of the low potential chain. In this same work (48), NQNO binding the Q_n site was found to further lower the midpoint of the c_n heme some 250 mV,

which would favor inhibition of *b*-heme oxidation. The experiments performed in the presence of 1 μ M FCCP (Figure 11) were designed to test whether FCCP and the subsequent reduction of the *b* hemes would have the same effect on state transitions as NQNO. FCCP inhibited state transition (Figure 11), and in further studies (not shown) inhibition of state transitions was found to be dependent on FCCP concentration. These results (Figure 11) show that reduction of *b*-hemes alone is sufficient to inhibit state transitions and is slightly recovered by addition of TDS to FCCP-treated cells. I also found that inhibition of state transitions by NQNO and FCCP is cumulative (data not shown) which suggests the inhibitor NQNO and the protonophore FCCP have similar consequences but different mechanisms of action. NQNO and FCCP both appear to cause changes in the midpoint potential of the c_n heme, and the subsequent reduction of the *b*-hemes correlated with state transition inhibition. This contributes to the hypothesis that the redox state of the low potential chain determines phycobilisome redistribution and also clarifies the effect of inhibitors on state transitions. Previous studies (41, 48) support the hypothesis that the redox status of the cytochrome *bf* complex low potential domain signals state transitions.

4.2.5 – Irreversibility of NQNO Effects on State Transitions Suggests

Permanent Chemical Modification or Tight Binding to the Cytochrome *bf* Complex.

The differential effects of NQNO under either oxidizing (addition in the light) or reducing (addition in the dark) conditions (Figure 10) suggest that NQNO when added under oxidizing conditions, may become modified or preferentially bind to a conformation of the cytochrome *bf* complex that does not inhibit state transitions. This

hypothesis is suggested by the irreversibility of the NQNO effect. When NQNO was added during darkness, the state transition was largely inhibited (Figure 10). If NQNO detaches from the cytochrome *bf* complex in darkness and then re-associates upon illumination, one would expect NQNO to inhibit state transitions as effectively when added during pre-illumination as when freshly added in the dark. Thus binding and substantial dissociation seems unlikely to occur as the added NQNO seems permanently unable to affect the state transition when added under oxidized (pre-illumination) conditions. The resulting hypothesis is that NQNO binds permanently in either case (cytochrome *bf* complex oxidized or reduced) but in different orientations or sites within the Q_n site. The binding orientation favored in the oxidized complex is conducive to state transition signaling whereas the orientation with NQNO bound to the reduced cytochrome *bf* complex is not. This hypothesis further suggests that there are conformational changes associated with the cytochrome *bf* complex in response to light, specifically affecting the Q_n site.

4.2.6 – Compiled Room Temperature Fluorescence Data and Implications.

My initial hypothesis that the cytochrome *bf* complex senses the redox status of the electron transport chain and thereby signals state transitions evolved from the failed prediction that TDS should inhibit the state transition. State transitions occurred despite apparent inhibition of the cytochrome *bf* complex by TDS. This suggested that contrary to expectations, elements upstream of the cytochrome *bf* complex Q_p site are not responsible for sensing changes in the redox status of the electron transport chain in the presence of light.

At the time of these experiments, cytochrome *f* re-reduction kinetics in the presence of TDS could not be investigated, though several other studies have shown TDS to inhibit at the Q_p site of the cytochrome *bf* complex (73). More recent experiments with a newly acquired Biologic JTS-10, Joliot-type Spectrophotometer allowed us to investigate the effects of NQNO and TDS on the cytochrome *bf* low potential and high potential chains. NQNO did reduce the *b*-hemes in *Synechocystis* PCC 6803 as expected, but TDS only marginally inhibited cytochrome *f* re-reduction kinetics at a concentration of 10 μ M, and only doubled the re-reduction half-time at a concentration of 50 μ M (data not shown). However, because TDS did show antagonistic effects on ROS production and in fluorescence experiments, the low level of inhibition from TDS in kinetic studies still represents a valid, observable effect. Note also that TDS inhibition may depend on whether cyanobacteria are grown in high (3%) or ambient (0.04%) CO₂ as indicated by recent studies with *Synechococcus* PCC 7002 (Krueger and Kallas, unpublished). These data further imply conformational changes occurring within the cytochrome *bf* complex in response to CO₂ availability and thus the redox status of the electron transfer chain.

My results showing NQNO inhibition of state transitions implicates the low potential chain as a redox sensor determining state transition signaling in the presence of light. Furthermore, the findings presented here support separate light and dark sensing/signaling mechanisms that mediate phycobilisome redistribution. As discussed above, the defining difference between the impacts of TDS and NQNO, in respect to electron transport chain redox imbalance, is the difference in redox status of the cytochrome *bf* complex low potential domain (reduced with NQNO, oxidized with TDS).

My findings therefore implicate the cytochrome *bf* complex as the site of redox sensing that determines phycobilisome redistribution in response to illumination.

The light-dependency of the NQNO-mediated inhibition of state transitions suggests that NQNO binding to the *cytochrome bf* Q_n site occurs when this site is in different orientations. The irreversibility of NQNO bound in the light (despite subsequent dark incubation) prior to state transition measurements suggests conformational changes occur at the Q_n site that mediate state transitions. Whether the redox sensing/signaling mechanism is the *b*-heme redox status, plastoquinone binding the Q_n site, or the conformation of the cytochrome *bf* complex, is unclear. In addition, the PetB-R214H mutant of *Synechococcus* 7002 still performs state transitions (Kallas, unpublished) as further evidence against the redox-alone hypothesis. However this mutation may permanently change the Q_n site to favor state transitions. Evolution may have found a place for each of these possible components in sensing and signaling mechanism of the state transition. For example, perhaps a conformational change induced by the light-induced excitation of the cytochrome *bf* chlorophyll is required to allow state transitions to occur via binding events at the Q_n site. Thereafter, the redox state of the low potential chain may modulate phycobilisome redistribution in the presence of light by gradually changing the conformation of the light-reacting cytochrome *bf* complex Q_n site, thereby changing association with other components (discussed further below) directly or indirectly affecting phycobilisome redistribution.

4.2.7 – 77K Fluorescence Spectra of *Synechocystis* PCC 6803 Cells Support Room Temperature Fluorescence Findings and Reveal Additional State Transition Components.

77K spectra provided an opportunity to view additional components involved in state transitions, specifically PS I (~720 nm), PS II (~680 nm), and phycobilisome fluorescence (~650-670 nm). Fluorescence from free or unassociated phycobilisome is more intense than fluorescence from attached or associated phycobilisomes. Free phycobilisome fluorescence was characterized in a study reporting phycobilisome fluorescence levels in PSI-less and phycobilisome-PSII deficient strains of *Synechocystis* 6803 (80). Phycobilisome fluorescence can be separated into two components: Fluorescence from associated phycobilisomes (~650 nm, primarily from phycocyanin) and fluorescence from unassociated/free phycobilisomes (~665 nm, originating primarily from allophycocyanin). Phycobilisome fluorescence spectra reported in PSI-less mutants show significantly higher fluorescence at 665 nm compared to 650 nm. Removing the ability of allophycocyanin to associate with PS II further increases the peak at 665 nm. These findings support the conclusion that fluorescence at 665 nm is primarily associated with unassociated phycobilisomes (80). Interestingly, ferredoxin NADP⁺ reductase (FNR) mutants also display this increase in the unassociated or phycobilisome-allophycocyanin peaks (665 nm) of phycobilisome fluorescence. FNR was found to localize near the phycobilisome core (81) and more recently (82) to be an NADP⁺ reductase. In contrast, the shorter form of Ferredoxin NADP⁺ reductase, which is not associated with phycobilisomes functions as an NADPH oxidase. Thus, normalizing the

77K spectra to ~650 nm emission (phycobilisome peak from mostly associated phycobilisomes) allows for comparison of the PSII, PS I, and unassociated phycobilisome peaks signal relative associated phycobilisomes (Figures 12-15).

On comparing the room temperature and 77K data, one might observe a discrepancy. In the room temperature fluorescence, there appears to be a decrease in PS II fluorescence upon addition of inhibitors such as NQNO or TDS. In the 77K data (Figures 12-15) there does not appear to be a decrease in PS II fluorescence after addition of the inhibitors in the dark; however, there is a decrease in PS I fluorescence. At room temperature, PS II fluorescence (~680 nm) and PS I (~720 nm) peaks are much broader and overlap, thus if PS II fluorescence does not decrease but PS I fluorescence decreases, the overall signal at room temperature will decrease. Room temperature studies (Figure 10) indicate that the inhibitors NQNO and TDS induce a decrease in fluorescence (680 nm) in the dark relative to the control. With 77K fluorescence (Figure 12), we see that this decrease observed in the room temperature studies can be attributed to a decrease in PS I fluorescence in response to NQNO and/or TDS. This evidence is key for understanding the mechanism of state transitions. The fact that TDS and/or NQNO have the same effect on phycobilisome redistribution in the dark (PSI fluorescence decrease, Figure 12), indicates that the sensing and signaling mechanism of this phycobilisome redistribution (disassociation from PS I in the dark) is not signaled by the redox state of the *b*-hemes. In previous room temperature experiments (data not shown) we saw a strong correlation between increasing FCCP concentration and slower rates of fluorescence reduction during dark incubation. This suggests that the sensing/signaling

mechanism of this state transition component (disassociation of phycobilisomes from PSI) is sensitive to other factors such as changes in pH or the proton gradient. PS I trimerization/monomerization has been described to occur in accordance with such changes (83) and I propose that this is the primary signaling factor of this state transition component.

I would not place the sensing and signaling mechanism of this state transition component (disassociation of phycobilisomes from PSI) upstream of the cytochrome *bf* complex because in the presence of either TDS or NQNO it is assumed that the plastoquinone pool will become more reduced. Phycobilisome disassociation from PS I is a state transition step that indicates that the oxidation of the electron transport chain is sensed. In the presence of TDS and NQNO oxidized electron carriers are expected only downstream of the cytochrome *bf* complex.

77K fluorescence spectra of cells treated with NQNO and/or TDS in the dark then frozen in the dark, or after exposure to light, showed that NQNO inhibited the state transition. Specifically, NQNO appears to inhibit phycobilisome association with PS II (Figure 14). Upon illumination, PS II fluorescence increased in the control and TDS-treated cultures. PS II fluorescence did not increase in the NQNO-treated samples. This result is consistent with the conclusions from the room temperature fluorescence data (Figures 10, 11). It appears that NQNO prevented the light-specific association of phycobilisomes with PS II and thus largely prevented the dark to light state transition.

77K fluorescence spectra of cells treated with NQNO and/or TDS in the light and then frozen in light or during subsequent dark incubation (Figure 15), show disruption in

the phycobilisome peak attributed to associated phycobilisomes (665 nm) compared to the control or TDS-treated cells. NQNO appears to force the peak downward, indicating that phycobilisomes are associating, but it is difficult to say whether these phycobilisomes associate with PS II or PSI. In room temperature experiments we see that fluorescence increases in NQNO cultures treated in the light (Figure 10); however, because the phycobilisome and PS I peaks overlap in this region (680 nm) at room temperature, one cannot conclude that phycobilisomes are associating with PSII. The 77K version of the experiment (Figure 15) indicates that some form of phycobilisome association is occurring in response to NQNO treatment, but without clear distinction to which photosystem. NQNO binds to the Q_n site of the cytochrome *bf* complex, and the effects of binding NQNO in the dark (Figures 10-15) have clear consequences (prevents state transitions and decreases PS I fluorescence). The finding that NQNO does have such a drastic impact on the 665 nm peak (from associated phycobilisomes) when added to cells in the light (Figure 15 Panel C), suggests that the cytochrome *bf* complex Q_n site must play a role in metering phycobilisome distribution. This is further evidenced by TDS in part, counteracting this NQNO-specific effect (Figure 15 Panel D), further indicating that the alteration of the 665 nm phycobilisome emission peak is specifically attributed to the low-potential domain or Q_n site of the cytochrome *bf* complex.

Therefore, I suggest that once light-induced conformational changes occur at the cytochrome *bf* complex, the modified *bf* complex interacts with proteins that associate with phycobilisomes, such as RpaC (59) or FNR (82). I suggest that this gradually affects the availability or efficiency of phycobilisome association with reaction centers based on

the redox status of the low potential domain of the cytochrome *bf* complex. Furthermore I suggest that these light-specific (or illumination-specific) conformational changes and associations are initiated by the chlorophyll embedded in the cytochrome *bf* complex.

4.2.8 – Characteristics of the Chlorophyll in the Cytochrome *bf* Complex.

In studies by Zito and colleagues (84), modification of residues near the chlorophyll within the cytochrome *bf* complex abolished state transitions in *Chlamydomonas reinhardtii*. In other studies (46), excitation of the chlorophyll correlated with reduction of the *b*-hemes suggesting an interaction between these components. Dashdorj et al. (45) showed the cytochrome *bf* complex chlorophyll excited state was quenched 20-25 times faster than that of chlorophyll in solution, suggesting active quenching mechanisms of the cytochrome *bf* complex. Crystal structures of the cytochrome *bf* complex reveal that the chlorophyll tetrapyrrole head and phytyl tail both emerge slightly from the cytochrome *bf* complex structure (Figure 16 B and C). In *Chlamydomonas reinhardtii*, subunit V (PetO), was found to be reversibly phosphorylated in response to state transition-inducing conditions and its ablation prevented state transitions (40). PetO was assumed to associate with the *bf* complex near the Q_p site (Ref. 36 & Figure 16A), which is right below the opening for the chlorophyll head.

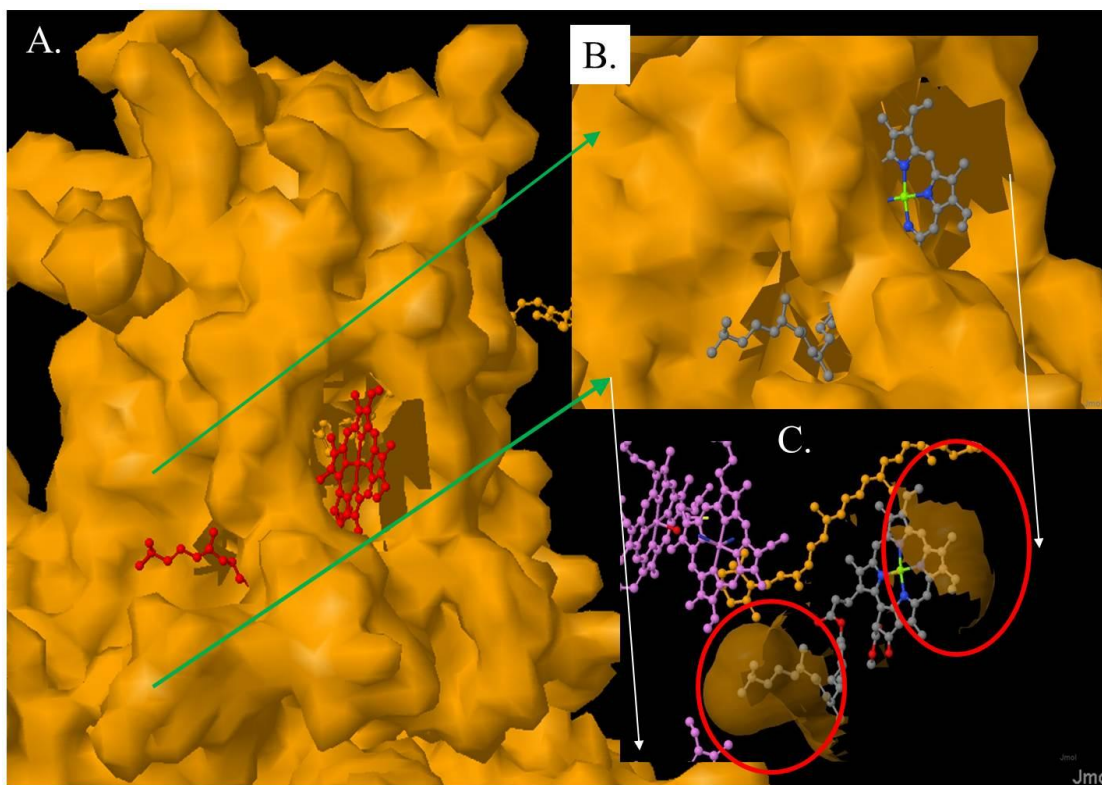


Figure. 16 – Cytochrome *bf* complex chlorophyll environment of *M. laminosus*. Imaged using Jmol-11.2.13: file: 2D2C.pdb.gz - <http://www.rcsb.org> – by Yan, Kurisu, and Cramer. The area view (A) shows the chlorophyll (red) protruding from the molecular ‘surface’ of the cytochrome *bf* complex. The inset (B) shows the location of the prosthetic group Mg (green). The solvent-exposed portions of the chlorophyll are shaded in the bottom-right inset (C).

4.2.9 – Mechanistic Roles for Excited Chlorophyll in the Cytochrome *Bf* Complex.

The cytochrome *bf* complex accepts electrons from plastoquinol regardless of whether its reduction occurs via succinate dehydrogenase, NADPH dehydrogenase, cyclic electron transport pathways, or PSII. If the *bf* complex is implicated in sensing the redox status of electron transport and mediating phycobilisome redistribution in response to light, it must have some means of determining when plastoquinol is being reduced by

light-mediated pathways such as PS II or cyclic electron transport. The presence of a chlorophyll molecule embedded in the complex might confer such photoreactivity.

The model proposed thus far is that the redox status of the *b*-hemes determines the distribution of phycobilisomes in cells exposed to light. If the chlorophyll passes energy to the *b*-hemes, then they should become more reduced leading to inhibition of the state transition according to my model. Physically the b_n (b_H) heme is too far away (16 angstroms) to participate in quenching of the chlorophyll. Practically, the chlorophyll is unlikely to add energy to the low potential domain, unless used to alleviate serial reduction shortages at the Q_n site. Thus it seems unlikely that the chlorophyll is used as an alternative energy source, not to mention that there is no obvious mechanism for its re-reduction. Moreover, the chlorophyll is probably not affected by the redox state of the low potential chain, as reduction of the *b*-hemes does not affect quenching of the chlorophyll (45). Thus it is unlikely that the chlorophyll acts as an energetic sink for the low potential chain as a means of removing excess energy. It has been proposed that a nearby tyrosine residue (Y105) may interact with the chlorophyll to quench its excited state (45). This would quench the chlorophyll excited state, but why have a chlorophyll at all if only to quench it?

If separate light/dark mechanisms exist to regulate the redistribution of phycobilisomes, then a key mediating sensor (cytochrome *bf* complex) must have some means of responding to the light stimulus. If a chlorophyll is used to respond to light then there must be some means by which this light-signaling molecule relays the signal without inducing damage through its excited state. In the cytochrome *bf* complex,

quenching of the chlorophyll excited singlet state occurs via the nearby tyrosine Y105 residue and very likely the carotenoid (45). Thus a protective mechanism is in place and the cytochrome *bf* chlorophyll appears to be well adapted to serve as a signaling molecule.

4.2.10 – Chlorophyll and Conformational Changes.

The Rieske iron-sulfur protein (ISP) of cytochrome *bc/bf* complexes undergoes large conformational during catalysis (85). Moreover, the ISP transmembrane region may also move during turnover of the *bf* complex (79). Perhaps chlorophyll excitation could be involved in changing the transmembrane environment, perhaps through excitation of the aromatic tyrosine residue mentioned above. Some modifications near the chlorophyll have disrupted dimerization of the *bf* complex (87)). Other reports indicate that state transitions are involved as a switch from linear to cyclic flow in *Chlamydomonas reinhardtii* (63). The cytochrome *bf* complex has not been convincingly isolated as an active monomer from *Synechocystis* 6803 nor any other cyanobacterium. Often in isolation from chloroplasts as well as cyanobacteria, the isolated dimer is many times more active than the monomer (86, 87)). Thus there is little evidence that the cytochrome *bf* complex exists as a monomer *in vivo*.

However, there is evidence from 2D electron microscopy that the cytochrome *bf* complex undergoes substantial conformational changes from one redox state to the next (79). The possible impacts of the trans-membrane conformational changes have, to my knowledge, not been the focus of empirical study. I propose that excitation of the chlorophyll initiates a movement of transmembrane helices that changes the Q_n site,

promotes the association of other components (discussed below), and the affinity of these components for the Q_n site is metered by the redox state of the *b*-hemes.

4.3 – Model for State Transitions in Cyanobacteria

4.3.1 - Defining the Skeleton of the Model.

There are two mechanisms by which phycobilisomes are believed to preferentially distribute harvested light energy to either PS I or PSII. Phycobilisome diffusion from one complex to another is involved in the “mobile phycobilisome” model (61). The “spillover” model of phycobilisome energy redistribution proposes that phycobilisomes remain more or less attached but distribute energy to either PS I or PS II because changes in the redox state of the electron transport chain induce conformational changes favoring excess energy to transfer from PS II to PS I or vice versa (18, 60, 47, 80).

State transitions occur in two basic steps. Step 1: disassociation of phycobilisomes or energy transfer from one photosystem and Step 2: re-association with the other type of photosystem. Thus the occurrence of even one of these two steps contributes toward a state transition because by disassociating or re-associating, the distribution of light energy would change the balance of electrons flowing through the electron transport chain. In this thesis, state transitions occurring in the dark will be designated ‘Dark Poise Transitions’ and State Transitions occurring in the light will be designated ‘Light Poise Transitions.’ State transitions occurring because of a change from light to dark or dark to light will be designated as ‘Dark Transitions’ and ‘Light Transitions,’ respectively.

The state transition cycles that I discuss will start with Dark Poise Transitions, named because mechanisms operate in the dark to poise the photosystems to make best use of the next illumination event. Upon illumination a Light Transition occurs allowing the Light Poise Transition to poise the distribution of light energy via phycobilisome association to balance the electron flow from PS II and PSI. When light is no longer present, a Dark Transition occurs allowing the Dark Poise Transition mechanisms to poise the system for another light event. The model revolves around the idea that there are two different mechanisms for redistribution of harvested light energy. One mechanism predominantly operates when light is present and the other when light is not present. These are poising phases named above. The switch between one mechanism and the other occurs when light is turned on or off. The transition from one mechanism to the other is termed Light Transition when the lights come on and a Dark Transition when the lights turn off.

4.3.2 – ‘Dark Poise’ State Transition Mechanism.

State transitions occurring in the dark (Dark Poise Transitions) or from darkness to light (Light Transitions) are slower than the opposite processes in the light (Light Poise Transitions) or in transitions from light to darkness (Dark Transitions). This suggests that these mechanisms are different or function differently in the dark than in the light. The oxidative potential (i.e. the potential to consume electrons) of the terminal respiratory oxidases, cytochrome oxidase and quinol oxidase, is significantly lower than that of PSI. Similarly, the respiratory plastoquinone pool reductive potential (i.e. potential to donate electrons) is significantly lower than that of PS II (26). Moreover, in our recent

experiments on P700 re-reduction kinetics, we have found cyclic electron transport to be a potent reducing force of the plastoquinone pool in *Synechocystis* 6803. If the redox state of the electron transport chain determines the relative state and association of the phycobilisomes then this ‘slower’ rate of respiratory chain electron transfer activity may be the primary reason for the slower rate of Dark Transitions and Dark Poise State Transitions.

4.3.2.1 - Hypothesis: I propose that Dark Poise State Transitions manifest themselves by increasing or decreasing the area atop PS I that binds phycobilisomes. Trimeric PS I provides the least phycobilisome-association area and monomerized PS I provides the greatest phycobilisome-association area per PS I reaction center. Thus, more trimerized populations of PS I will have lower phycobilisome-mediated excitation of PS I fluorescence than highly monomerized populations. Zhao et al. (83) found that in *Spirulina platensis*, the oligomeric state of PS I is determined by the local pH of the cytosolic side of PS I complexes, with low pH inducing monomerization. Therefore I hypothesise that this pH-induced change in oligomeric state of PS I is responsible for the changes in fluorescence observed in the dark in response to changing redox states of the electron transport chain.

4.3.2.2 –A state transition mechanism that depends simply on light on and off signals. Recent investigation into the mechanism of state transitions, by Zhao et al. (83) in *Spirulina platensis*, suggested there are separate mechanisms regulating the process and that both models (mobile redistribution and spill-over) of phycobilisome redistribution are valid. A related work additionally showed that the relative contributions

of these mechanisms are quantifiable (88). *Spirulina platensis* is unique in that PS I monomers and trimers fluorescence at distinctly different wavelengths, 730 nm and 760 nm, respectively. Following the changes in fluorescence at these peaks in dark to light, changing light, an light to dark transitions allowed Zhao et al. (83) to understand how oligomerization changed in regard to the state transitions. The chemical, betaine, was found to affix phycobilisomes to the membrane, thereby inhibiting phycobilisomes from diffusing and thereby eliminating the contribution of mobile phycobilisomes to the state transition. The remaining changes in fluorescence of the photosystems in response to dark-light or light-dark transitions were unaffected by this chemical. Only Light Transitions (dark to light) and Light Poise Transition were affected by betaine, suggesting that only the mechanism responding to changes in light intensity induced a response that required diffusion of phycobilisomes.

The fact that betaine does not affect Dark Poise Transitions suggests that photosystems perform the primary conformational changes inducing preferential redistribution of the energy harvested by the phycobilisomes. Zhao et al. (83) describes both mobile and spillover contributions to the Light Transition and that this contribution switches to primarily mobile phycobilisome-mediated redistribution of harvested light energy during Light Poise Transitions.

Zhao et al. (83) concludes that the PSI-local cytosolic pH determines oligomerization. He explains that PSI-trimers are held together by the electrostatic interactions of PsaD in the cytosol (83). In his experiments he showed that as the pH decreased on the cytosolic side (n-side), trimers became less likely to be formed during

Dark Poise Transitions. This suggests that fewer protons on the cytosolic side favors trimerization and more protons favors monomerization (83) Zhao et al. (88) further concluded that the electron transport chain determines mobile-phycobilisome association. I propose that the Dark Poise State Transition mechanism might occur via the spillover model in which state transitions occur through oligomeric state changes that allow more or less energy transfer from phycobilisomes to PSII. Additionally the remaining mechanisms, Light Transitions and Light Poise Transitions, could be synonymous with the mobile phycobilisome model regulated primarily through changes in the cytochrome *bf* complex.

4.3.2.3 – The characteristics of CpcG1 and CpcG2 phycobilisomes. Kondo et al. (89) found two forms of phycobilisomes in *Synechocystis* 6803. CpcG1 contains an allophycocyanin core and associates with trimeric PSI. Another form, CpcG2, found to lack allophycocyanin core proteins, associates tightly with thylakoid membranes and has three times the energy transfer capacity to PS I relative to CpcG1. The characteristics of these phycobilisomes lend themselves well to being the physical mediators of the mobile phycobilisome and spillover models of phycobilisome-mediated energy redistribution. CpcG1 is only loosely associated with the membrane and favors trimeric-PSI complexes (present primarily in the light). This suggests that this more hydrophilic phycobilisome complex diffuses between PS II and trimeric-PSI and functions in the mobile phycobilisome model. The CpcG2 phycobilisomes associate tightly with the membrane and favor energy transfer to PS I, suggesting that this form of phycobilisome might facilitate the spillover mechanism of energy distribution. The CpcG2 variety of

phycobilisome, less mobile and anchored as it is to the membrane, likely forms associations with the photosystems through the dynamics of the photosystems themselves. Thus if PS I monomerizes more, the CpcG2 phycobilisome excitation energy transfer can be passed more readily to PS I because more PS I complexes can interact with the CpcG2 phycobilisomes. Kondo et al. (89) assessed the efficiency of phycobilisome transfer under dark conditions, primarily involving monomerized PS I (88), and concluded that under these conditions, CpcG2 has a three times greater rate of energy transfer to PS I than CpcG1. Thus the CpcG2 phycobilisome provides a physical basis for the spillover model of energy redistribution primarily mediated by the oligomerization state of PSI.

Kondo et al. (89) report that CpcG1 and CpcG2 phycobilisomes are widespread among cyanobacteria. They note that in *Synechococcus elongatus*, the organism favored in the studies of Mullineaux (60) for their predictable state transitions, only the genes for CpcG1 are present in the genome. However, NCBI GeneBank does show sequences for CpcG1 and CpcG2 genes in the genome of *Synechococcus elongatus*. In hydropathy plots of CpcG1 and CpcG2 C-terminal sequences from *Synechocystis* 6803 and *Synechococcus elongatus* (Figure 17), only the CpcG2 from *Synechocystis* 6803 displays a significant hydrophobic region at the C-terminus correlated with the CpcG2 PSI-specific activity described in Kondo et al. (89). This further correlates well with the conclusions by Joshua and Mullineaux (60) suggesting that diffusion is required for state transitions. This is likely the case if an organism only uses CpcG1-like phycobilisomes. State transitions assessed in *Spirulina platensis* by Zhao et al. (83, 88) came to a different conclusion

despite using a similar method to limit the diffusion of phycobilisomes. I conjecture that this is because *S. platensis* has both the CpcG1 and CpcG2 types of phycobilisomes, which was not addressed in Zhao et al. (83). Genome sequences of *Spirulina* (*Arthrospira*) species are now available, which could help answer this question.

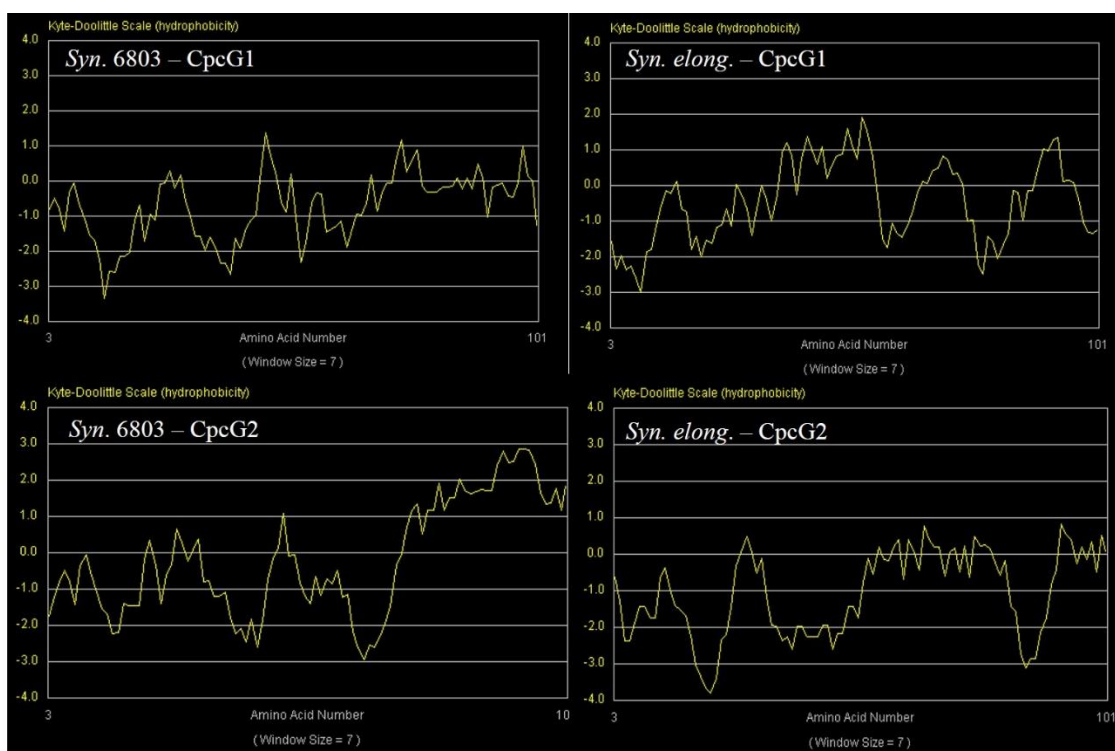


Figure 17. – Hydropathy plots of the last 100 amino acids of the C-termini of the CpcG1 and CpcG2 phycocyanin proteins of *Synechococcus elongatus* and *Synechocystis* PCC 6803. A Java-based hydropathy program, by R. A. Bowen from Colorado State University, was used to analyze these sequences (<http://www.vivo.colostate.edu/molkit/hydropathy/index.html>) using the Kyte-Doolittle hydropobicity index. (116)

CpcG2 phycobilisome is a primary candidate to explain the physical manifestation of the spillover model. This variety of phycobilisome might also be the physical basis of the Dark Poise State Transitions described in this work (Section 4.3.1.1). Thus, oxidation of the cytochrome *bf* high potential chain or other downstream changes induced by slower turnover of the *bf* complex, could initiate trimerization of PSI. This would decrease the

number of PS I complexes capable of associating with CpcG2 phycobilisomes, allowing more energy from these phycobilisomes to flow to PSII. This would be observed as a decrease in PS I fluorescence and an increase in PS II fluorescence. However because, as mentioned by Kondo et al. (89), CpcG2 has a greater efficiency of energy transfer to PS I, the increase in PS II fluorescence would not as great as that induced by light.

4.3.2.4 – Conclusions regarding pH-dependent PS I oligomerization and CpcG2 phycobilisomes as primary components of the Dark Poise State Transition mechanism.

The Dark Poise Transition mechanism senses the redox status of the electron transport chain down-stream of the cytochrome *bf* complex. This is evidenced by data presented here (Figures 10 & 15) from *Synechocystis* 6803, and is supported by the conclusions of Zhao et al. (83) with *Spirulina platensis* and Kondo et al. (89) with *Synechocystis* 6803. These conclusions provide a rational framework for interpreting the 77K (Figures 12-15) and room temperature fluorescence data (Figures 10 and 11) presented here.

In cells exposed to light, PSI-trimers are predominant and favored by CpcG1 phycobilisomes, which possess specificity for PSI-trimers but are far less efficient at transferring their energy to PS I than CpcG2 phycobilisomes. On Dark Transition PS II stops reducing the electron transport chain, thus the pH near the thylakoid begins dropping as protons accumulate on the n-side of the thylakoid membrane via ATPase. According to Zhao et al. (83) this drop in pH destabilizes PS I trimers creating a predominantly PSI-monomer population. As the PS I trimers monomerize, CpcG1 phycobilisomes disassociate. Thus, during Dark Transitions, phycobilisome energy transfer shifts from two CpcG1 phycobilisomes transferring energy among three PS I

complexes during illumination to three CpcG2 type phycobilisomes transferring energy to monomers at a 1:1 ratio in the dark. The observed effect of which increases PS I fluorescence as a result of the Dark Transition.

In room temperature fluorescence traces (Figure 10), fluorescence drops quickly at first when dark incubation begins and then continues to level out toward some redox poise. I propose that the rapid drop is due to the inactivation of Light Poise Transition mechanisms (discussed below in Section 4.3.3 – 4.3.4) and that the more gradual changes are due to CpcG1 phycobilisomes detaching from monomerized PS I trimers and the slower association of CpcG2 phycobilisomes with PS I monomers. This is observed as a net decrease in fluorescence because the filter for the room temperature studies measured primarily PS II fluorescence (at ~ 680 nm), and is only marginally counteracted by increased fluorescence from detached CpcG1 phycobilisomes (~ 670 nm) and PS I association of CpcG2 phycobilisomes (~ 720nm).

Observed effects of TDS and NQNO are also consistent with the conclusions of Zhao et al. (83) regarding pH-dependent oligomerization. In the dark, the electron transport chain of cyanobacteria is primarily reduced and according Zhao et al. (83) the n-side of the thylakoid membrane would have a low pH promoting monomerization of PS I and a higher PS I fluorescence due to the 1:1 ratio with associated CpcG2 phycobilisomes. The redox potential of the PQ pool during darkness is maintained in *Synechocystis* 6803 primarily by NDH and SDH reduction of the plastoquinone pool, and its oxidation by quinol oxidase and the cytochrome *bf* complex. Of these complexes, only the cytochrome *bf* complex translocates protons for use by ATPase. Blocking the

cytochrome *bf* complex with inhibitors makes protons on the p-side limiting and quinol oxidase continues to consume protons from the p-side by forming water, subsequently raising the pH on the p-side. When TDS and/or NQNO slow turn-over of the cytochrome *bf* complex to some degree, the effect is fewer protons added to the p-side for ATPase to use, thus fewer protons flow back to the cytosolic side. The result is an increase in pH on the n-side of the thylakoid subsequently inducing trimerization of PS I complexes

4.3.3 – Mechanism of ‘Light Transition’ State Transitions.

4.3.3.1 - Hypothesis: I hypothesize that the mechanism for the Light Transition (dark to light) is the formation of a binding-platform atop cytochrome *bf* complex dimers comprised of the protein Ssr2998 bound at the Q_n site and its association with PetM in response to excitation of the chlorophyll embedded in the cytochrome *bf* complex. I suggest this complex associates with the rods of phycobilisomes and provides stability for their association with PS II via the RpaC protein.

4.3.3.2 – The PetM subunit of the cytochrome *bf* complex and its role in phycobilisome redistribution. Schneider et al. (90) describe a *petM* knock-out mutant of *Synechocystis* 6803. PetM is a small hydrophobic protein that has been shown to co-purify with the cytochrome *bf* complex (91). Schneider et al. (90) show that disruption of the gene encoding PetM results in decreased PS I and phycobilisome content. If PetM does not directly regulate transcription of PS I and phycobilisomes, it likely affects their composition indirectly. Photosystem composition and phycobilisomes has been shown to be regulated by the redox status of the electron transport chain (17, 92). Thus if phycobilisome and PS I content has decreased, this indicates that over-oxidation of the

electron transport chain has occurred and the organism has compensated by decreasing the activity of PSI. Indeed, when Schneider et al. (90) tested cytochrome *f* re-reduction kinetics, they found that the PetM knock-out mutant displayed significantly reduced re-reduction kinetics relative to the wild-type. However these slower kinetics were only observed if PS II was the sole source of reduction for the electron transport chain.

Schneider et al. (90) also used variable fluorescence kinetics measurements to determine the redox state of the plastoquinone (PQ) pool, which lies between PS II and the cytochrome *bf* complex. But using DBMIB to block the cytochrome *bf* complex, they observed that the mutant could not reduce the PQ pool even when glucose was added to stimulate NADH mediated reduction. Ultimately they were able to achieve full reduction of the PQ pool by blocking quinol oxidases with the inhibitor PCP (pentachlorophenol). They concluded that deletion of PetM resulted in increased production or activity of the quinol oxidases.

Alternatively, the results of Schneider et al. (90), together with those from the original paper on the PetM- mutant (93), and a later paper on cytochrome *bd* oxidase (84), may be interpreted differently. I believe that decreased association of PBS with PS II and subsequent decrease in plastoquinone pool reduction is responsible for the over-oxidation observed in the variable fluorescence results of Schneider et al. (90). The original characterization by Schneider et al. (93) suggested that the plastoquinone pool was over-reduced because state transitions were perturbed. These studies were done with room temperature, pulse amplitude modulated fluorescence kinetics using saturating actinic flashes superposed over continuous, low intensity background illumination (93).

Additionally 77K fluorescence spectra from these studies show different PSII:PSI fluorescence ratios which could be attributed to different culture conditions or perhaps evolution of the PetM mutant during several years in laboratory culture. If we assume that the PetM mutant retained its original characteristics (perturbed phycobilisome association with PSII), then we would expect to see a lower PSII:PSI ratio compared to the wild type because, Hihara et al. (17) showed that transcription of the PS II D1 gene increased in response to DCMU and DBMIB treatment, but a significant reduction in phycobiliprotein gene transcription occurred in response to the same condition. Thus over a protracted period of over-oxidization of electron carriers downstream of the cytochrome *bf* complex, a primary component of PS II is up-regulated and phycobiliproteins are down regulated. This may produce a 77K spectrum similar to the one shown by Schneider et al. (90).

Additionally, the original paper (93) reported an over-reduced electron transport chain upstream of the cytochrome *bf* complex due to slower turnover of the *bf* complex. Slower turnover of the *bf* complex was also observed by Schneider et al. (90) in cells under photoautotrophic conditions. But these authors propose that the plastoquinone pool is more oxidized in the PetM mutant because F max levels of variable fluorescence were not reached under lighted conditions in the presence of DBMIB. The PetM mutant did reach F max fluorescence when supplemented with glucose in the presence of DBMIB and PCP. Schneider et al. (90) conclude that an overactive quinol oxidase in the PetM mutant is responsible for the oxidized state of the PQ pool, and that this explains the necessity of adding PCP to reach F max fluorescence levels. However, a subsequent

study from the same group under similar conditions showed that wild type *Synechocystis* 6803 as well was unable to reach F max levels (84). Therefore, I cannot reach the same conclusion that Schneider et al. (90) reached.

I agree the Schneider et al. (90) data shows the plastoquinone pool to be more oxidized, but I suggest that this is due to reduced energy transfer to photosystem II from phycobilisomes, as shown in the 77K spectra and room temperature fluorescence kinetics reported in Schneider et al. (93). To test this hypothesis, 77K spectra of the PetM mutant should be compared in state 1 (PQ pool oxidized) and state 2 (PQ pool reduced) conditions relative to the wild type. This way one can determine whether phycobilisome attachment is normal or aberrant in this mutant. Additionally, the variable fluorescence experiments in the PetM mutant treated with glucose and PCP should include the wild type for comparison.

The conclusion I draw from these studies is that the PetM subunit of the cytochrome *bf* complex is involved in stabilizing phycobilisome/cytochrome *bf* interactions that facilitate in proper association of phycobilisomes to PSII.

4.3.3.3 – The new cytochrome *bf* complex subunit Ssr2998. A 7.2 kDa soluble protein, corresponding to the open reading frame *ssr2998*, was found to be copurified with the cytochrome *bf* complex in *Synechocystis* 6803 (91). The work by Volkmer et al. (91) describes a knockout mutant of *Ssr2998* as having an increased PSI:PSII ratio and unable to adapt to high light or mixotrophic conditions due to impaired cytochrome *bf* complex turnover and subsequent over-reduction of the plastoquinone pool. This conclusion is evidenced by cytochrome *f* and P700 re-reduction kinetics being 1.7 times

slower in the mutant and impaired growth under conditions which increase reduction to the plastoquinone pool at light intensities greater than low light (20 μ E) under autotrophic growth conditions. Further, this over-reduced plastoquinone pool was deemed the explain why the mutant did not appear to exhibit characteristic changes in photosystem fluorescence during dark to light transitions associated with state transitions. This was evidenced by the reappearance of a state transition in the Ssr2998 mutant pre-treated with PPBQ – a chemical which oxidizes the plastoquinone pool. However, the state transition made possible by the addition of PPBQ was still significantly smaller than in the wild type.

In the data presented by Volkmer et al. (91), we see that the state transition of the mutant, at the level of PSII, does increase in the dark after treatment with PPBQ, as it does in the wild type, but when illuminated, the increase in PS II fluorescence of the mutant is far less than in the wild type. However, PS I fluorescence does continue to decrease on illumination in the mutant treated with PPBQ. Thus, I agree that the plastoquinone pool is over-reduced and likely exerts pressure on the state transition mechanism. However, I interpret the reduced PS II fluorescence change as an impaired association of phycobilisomes with PSII. If the plastoquinone pool over-reduction was alleviated by PPBQ, then PS II fluorescence should have reached wild type levels. Additionally, the primary structure of Ssr2998 suggests a soluble protein, thus it may be soluble on the N-side aqueous phase of the membrane and associate with the cytochrome *bf* Q_n site.

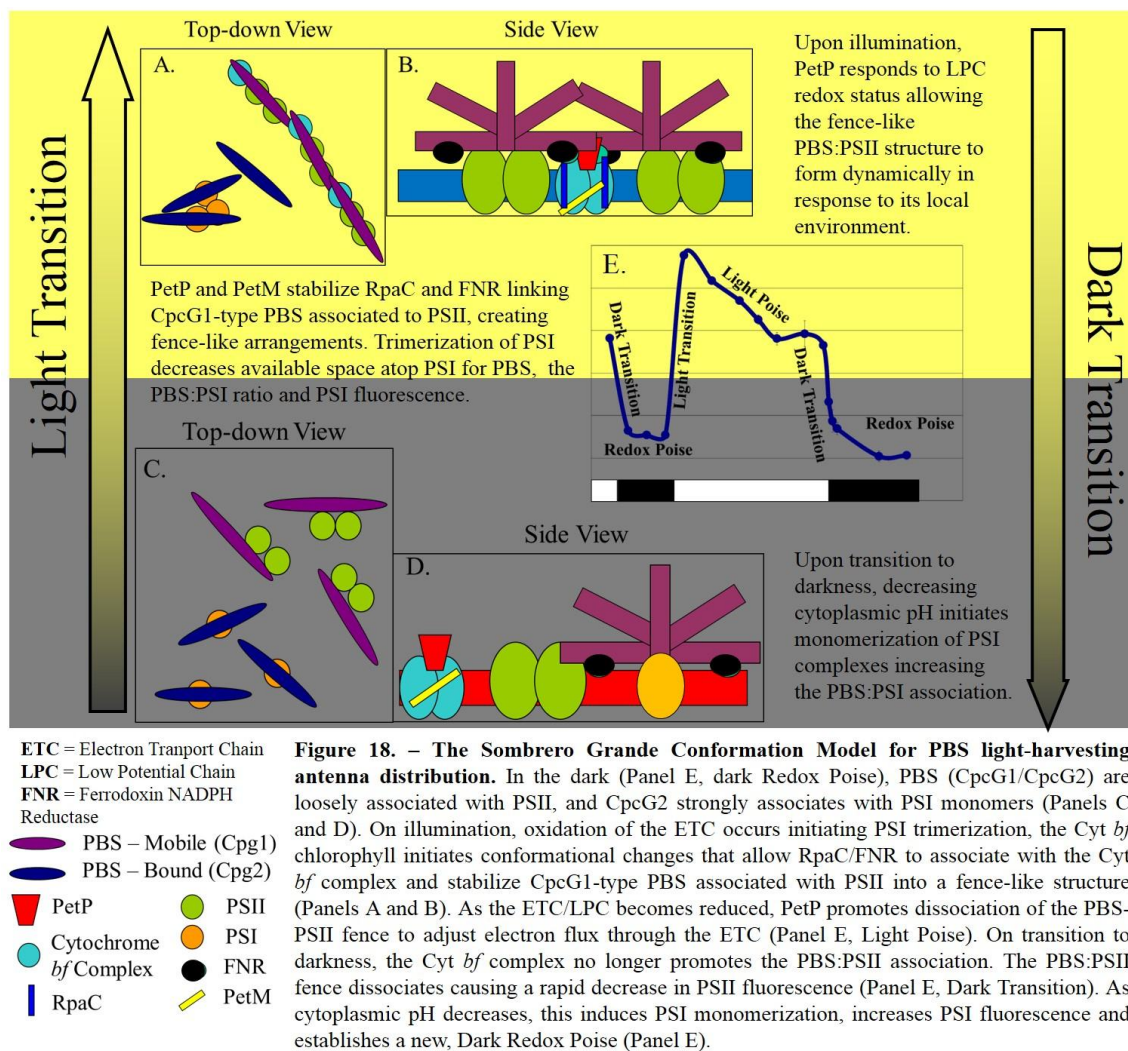
My conclusion is that Ssr2998 is a soluble protein that binds in the Q_n site and upon illumination serves to associate with phycobilisome rods. Its absence or perturbation in binding the Q_n site (e.g. via addition of NQNO) impairs reduction of plastoquinone at the site and slows the overall turnover of the complex causing reduction of the *b*-hemes. I suggest that Ssr2998 primarily associates with the *bf* complex in the light and its association is mediated by PetM (90), which responds to excitation of the chlorophyll embedded in the cytochrome *bf* complex.

4.3.3.4 - The *RpaC* mutant. The *RpaC* mutant of *Synechocystis* 6803 is a knockout of a small 9 kDa protein that contains two transmembrane helices. The *RpaC* mutant appears to be stuck in state 1 (PQ pool oxidized) (59). The *RpaC* mutant does not appear to perform state transitions via phycobilisome energy transfer as measured by 77K fluorescence after phycobilisome-excitation (580 nm) nor by variable fluorescence measurements at room temperature (59). However, upon excitation of chlorophyll (480 nm), 77K fluorescence emission spectrometry does show changes in photosystem fluorescence suggesting the spill-over mechanisms, as described by Zhao et al. (83) in *Spirulina platensis*, are still at work in the *RpaC* mutant. A related paper (94) discusses the re-introduction of a functional *rpaC* gene, controlled by a constitutive *psbAII* promoter, into the $\Delta RpaC$ background. Mullineaux et al. (94) found that this *rpaC* overexpression mutant was still defective in state transitions as determined by 77K fluorescence experiments. The overexpression mutant had a high PS II to PS I ratio suggesting that increased expression of PS II occurred to compensate for over-oxidation of the plastoquinone pool. Mullineaux et al. (94) come to the conclusion that *RpaC* is a

membrane protein that attaches to phycobilisomes near the APC core and serves to stabilize PSII/phycobilisome interaction. These authors reason that overexpression of RpaC causes phycobilisomes to become less mobile because the number of RpaC attachment points out-numbers PS II complexes.

I propose that RpaC and the cytochrome *bf* complex work together to associate phycobilisomes with PSII. RpaC associates phycobilisomes to PS II and the cytochrome *bf* complex associates phycobilisomes to one-another forming the rigid, fence-like arrangement described in Kuhl et al. (95) and originally in Morschel et al. (96). In the Δ RpaC mutant, no movement occurs because association between PS II and phycobilisomes is transient and unstable without RpaC. Therefore I speculate that whether phycobilisomes are strung together by cytochrome *bf* complex dimers, or not via the actions of Ssr2998 and PetM, the row-like arrangement of phycobilisomes would be inconsequential without a solid foundation for the phycobilisome-fence to associate with PSII. Mullineaux et al. (94) suggest that when RpaC is overexpressed, multiple RpaC molecules block proper association of phycobilisomes to PS II by “clogging” the binding interface. As a result, transfer from phycobilisomes to PS II is significantly perturbed. The resulting over-oxidation would signal increase in PS II production and decrease in CpcG2-type phycobilisomes as described for oxidative conditions in Hihara et al. (17).

4.3.4 – Proposed State Transition Cycle: The Sombrero Grande and Conformation Model of State Transitions.



4.3.4.1– In the Dark. In the dark, phycobilisomes are primarily associated with PS I in a mix of monomers and trimers. Kondo et al. (89) explains that under these conditions the CpcG2 phycobilisomes are primarily associated with PS I monomers (Figure 18 Panel C) and the CpcG1 phycobilisomes associate with the trimeric PS I complexes. In the dark the electron transport chain is primarily reduced because

respiratory electron transport chain components such as NADPH dehydrogenase and succinate dehydrogenase keep the plastoquinone pool moderately reduced (26). In the dark, the local pH of the n-side of the thylakoid membrane is low because I speculate that the cytochrome *bf* complex maintains a steady supply of protons for ATPase to push back across the membrane and the primary contributor to plastoquinone pool reduction is succinate dehydrogenase, which does not directly impact the local pH as succinate is derived through the citric-acid cycle (26).

On oxidation of the electron transport chain by PS I, local increases in pH on the n-side of the thylakoid membrane induce trimerization of PS I, causing the PS I monomer:trimer ratio to shift toward trimers. As a result more CpcG1 phycobilisomes associate with PS I trimers. However, because of limiting space on the top of trimeric PS I, fewer CpcG1 phycobilisomes than CpcG2 phycobilisomes can associate. CpcG2 phycobilisomes appear to contribute slightly to PS II under these conditions but are rather inefficient at energy transfer to PS II (Ref. 89 and this thesis Figure 13). One might also think that because the CpcG1 phycobilisomes associate preferentially with trimeric PS I, that PS I fluorescence would increase contrary to the observed decrease in response to these oxidizing conditions (light, TDS and/or NQNO inhibition, or PS I oxidation of the electron transport chain). However, the monomer to trimer transition is essentially swapping a phycobilisome per monomer for one or two phycobilisomes per trimer. The net result is a decrease in PS I fluorescence shown here (Figure 12) and elsewhere (83). Further this is supported by the finding that energy distribution within PSI-trimers is asymmetric (97). This suggests that within a PS I trimer one monomer is the principle

oxidizer and over-flow energy is funneled to the other two monomers in the trimer with equal probability. Thus, I propose the steric hindrance atop PS I trimers induces PS I fluorescence decreases as observed in the dark (Figure. 12) after treatment with TDS and/or NQNO, and upon illumination (Figure 14) in response to the oxidizing conditions that increase pH on the n-side of the membrane.

4.3.4.2– Upon Illumination. Upon illumination, the phycobilisomes, that do not fit atop trimeric PS I, associate with PS II via the Light Transition (Section 4.3.3, Figure 18, Panels A,B, and E) mediated by the cytochrome *bf* complex. According to Mullineaux et al. (94), on illumination RpaC proteins allow phycobilisomes to properly bind PS II complexes during state transitions. Excitation of the chlorophyll within the cytochrome *bf* complex and the subsequent conformational change modifies the association of Ssr2998 (hereafter PetP as the name proposed in 91) and PetM. I suggest that PetP associates near the Q_n site of the cytochrome *bf* complex and serves to ‘hold’ floating phycobilisomes via interaction with the ferredoxin NADP reductase (FNR) attached to phycobilisome rods (82). PetM, which protrudes from the two ends of the cytochrome *bf* dimer, then associate with RpaC molecules associated with the phycobilisomes atop PSII. One end of the cytochrome *bf* dimer is attached, via PetP to the CpcG1 phycobilisome (which is attached to PS II via RpaC) and via PetM interacting with RpaC (Figure 18 Panel B). The other end of the cytochrome *bf* dimer awaits a second phycobilisome-RpaC-PSII complex. Once associated, the cytochrome *bf* complex forms a link between one phycobilisome-RpaC-PSII complex to a second phycobilisome-RpaC-PSII complex, forming a rigid row-like chain or fence. The subsequent changes in

redox state of the cytochrome *bf* complex low-potential chain, change the relative affinity of PetM for RpaC, and PetP for FNR. The more oxidized the cytochrome *bf* complex becomes, the stronger the PetP-FNR and PetM-RpaC interactions become, creating larger/longer phycobilisome fences (Figure 18, Panels A and B). The gradual increases or decreases in size of the phycobilisome “fence”, I propose are the physical manifestation of the Light Poise Transitions (Figure 18, Panel E) that serve to adjust phycobilisome distribution under illumination that is continuous but changing in light composition or intensity.

4.3.4.3– Model Discussion and Criticisms. The Light Transitions have specific characteristics (4.3.3) and likely include PS I oligomeric change components (83, 88) and mobile phycobilisome mechanisms (83, 88, 89). The 77K data presented in this thesis show that PS II fluorescence increases more during Light Transitions (Figures 14-15) than in Dark Poise Transitions (Figures 12-13) in response to inhibitors. This seems reasonable considering the differences in electron transport rates between dark respiratory electron flow and photosynthetic electron transport in the light. However, data shown in Figure 14 indicate that the fluorescence changes relative to PS I induced in the dark by inhibition of the cytochrome *bf* complex appear equivalent to those induced by illumination. This suggests that some maximal fluorescence change is already induced by PSI-specific mechanisms in the dark. However these PS I extremes are not reciprocally reflected by PS II fluorescence extremes. Thus the redox changes induced by cytochrome *bf* complex inhibition in the dark fully induce state transitions relative to PS I but not those relative to PSII. This suggests that the Light Transition components involving the

mobile phycobilisome components likely do not function in the Dark Poise Transition to any great extent. The Light Transition on the other-hand involves both mechanisms. This may have been seen in the Zhao et al. study (83) but they had not forced redox extremes in the dark with cytochrome *bf* complex inhibitors. In the light, the mobile phycobilisome components primarily function through cytochrome *bf* complex-mediated Light Transition and Light Poise Transition mechanisms. The PS I oligomerization, and thus Dark Poise Transitions, likely also function in the background during Light Poise Transitions. One can imagine that the changes in pH required to force PS I to trimerize have already occurred with the presence of light because the higher oxidative potential of the photosynthetic complexes quickly raises the local pH on the n-side of the membrane. If ATPase runs at a consistent rate (limited by available protons on the p-side, and the steepness of the transmembrane ΔpH gradient), then the n-side will remain at a more alkaline pH in the light, meaning PS I complexes remain trimerized. However, at very low light intensities, where the proton translocation by ATPase could restore a lower pH to the n-side, against a slower PSII/cytochrome *bf* complex-mediated proton translocation to the p-side, one might observe Dark Poise Transition mechanisms at work. Thus, low-light experiments provide a means of testing this hypothesis. During Dark Transition the faster component of phycobilisome redistribution likely ‘resets’ via the Light Transition and Light Poise Transition components dissociating and freeing phycobilisomes from the rigid row-like organization associated with PS II (95).

Putting these mechanisms into perspective suggests that the Sombrero Model functions to regulate CpcG1 and CpcG2 attachments to PSI. These in turn are regulated

by cytochrome *bf*, high-potential chain oxidation and/or changes in cytosolic pH during dark poise transitions and light transitions. Association of CpcG1 phycobilisomes with trimeric PS I and CpcG2 phycobiliproteins with monomeric PS I are the defaults. In a sense they regulate themselves with the changing oligomeric state of PSI. The light-dependent mechanisms of the cytochrome *bf* complex serve to modulate phycobilisome associations with PS II through two mechanisms. The mutants (Δ PetM and Δ Ssr2998) show the transient effect of the Light Poise Transition (absent in Δ RpaC) but they cannot maintain wild type rates of cytochrome *f* or P700 kinetics in the light. Schneider et al. (90) showed that the slower electron transfer kinetics of Δ PetM were not observed in the dark. The PS I and phycobilisome compositions are lower in this mutant consistent with an electron transport chain starved for PS II reduction potential. Further, inhibition of quinol oxidases with pentachlorophenol allowed variable fluorescence induction comparable to that seen after DCMU treatment, suggesting that the plastoquinone pool remained in a more oxidized state in the mutant. Schneider et al. (90) suggest this occurs because of an over-active quinol oxidase. Alternatively, I believe their data could indicate that the Δ PetM mutant is impaired in capturing sufficient light energy for PSII.

The Δ Ssr2998 (PetP) mutant appears to have similar kinetics to the Δ PetM mutant except that their plastoquinone pool redox potentials are shifted in opposite directions, more reduced and more oxidized, respectively. For reasons described above (4.3.3.3), The Ssr2998 protein is required for efficient function of the cytochrome *bf* complex in the light, and may inhibit turnover of the *bf* complex by backing up the low potential chain in a manner similar to the PetB-R214H mutation (34) and by addition of NQNO. However,

the absence of Ssr2998 (PetP) impairs association of phycobilisomes with PS II (4.3.3.3). Thus PetP may act as a platform for attaching phycobilisomes and *cytochrome bf* complexes to PS II via ferredoxin NADP reductases bound to phycobilisomes (82 and Figure 18). PetM, located on the periphery of the *cytochrome bf* complex and adjacent to the chlorophyll-quenching β -carotene (45) may be sensitive to shifts in conformational changes induced by the excitation of the chlorophyll molecule. Thus, PetM may be responsible for ‘holding onto’ RpaC molecules attached to phycobilisome rods and by stringing dimeric *cytochrome bf* complexes together helps form a *cytochrome bf* dimer-phycobilisome ‘fence.’ Subsequent changes in the redox state of the *cytochrome bf* complex add to or reduce the size of this ‘fence’ through the activity of RpaC.

Hihara et al. (17) looked at global gene expression in *Synechocystis* 6803 after transition to high light intensity. They found that expression of the *rpaC* transcript significantly dropped off within the first 15 minutes. Mullineaux and Emlyn-Jones (61) concluded that RpaC was not essential and only optimized light-harvesting efficiency at very low light intensities. In this context, Hihara’s findings make sense (17). Association of phycobilisomes with PS I is a type of safety net for photosynthetic electron transport. I suggest there is thus a selective pressure for phycobilisomes to associate with PSI. The Sombrero Grande *Cytochrome bf* Phycobilisome Fence Model assumes that phycobilisome association with PS I is a default ‘setting’ and thus state transitions have evolved to provide energy to PSII. In this way ‘relaxation’ or disruption of state transition components that do not affect electron flux directly (such as PetM and RpaC, and unlike Ssr2998) tend to generate more oxidized electron transport chains. This makes sense

from a photoprotective standpoint because having excess excitation energy and reduction potential flow into the electron transport chain from PS II may cause severe problems in the form of overproduction of reactive oxygen species. In this context, Hihara's data (17) also make sense because after exposure to high light, down-regulation of *rpaC* would be important if it does indeed facilitate association of phycobilisomes to PSII.

In studies measuring the effects of phycobilisome and photosystem stoichiometry in *Porphyridium cruentum* in response to growth under varying light conditions (98), one can see that PS II is preferentially down regulated in response to green light, primarily absorbed by both photosystems. Compared to red light, the distribution of PSI:PSII in green light is significantly skewed toward PSI. If state transitions play no photo-protective purpose as suggested by Mullineaux (61), then why is PS II being down-regulated relative to available phycobilisomes under conditions that are preferential to it? Why down-regulate a PSII-specific phycobilisome association protein like RpaC? I think the answers to these questions imply a photo-protective effect of state transitions as suggested by Hihara's group (17).

The high-light ecotype of *Prochlorococcus marinus* MED4 (which uses chlorophyll *a/b* light harvesting proteins rather than phycobilisomes) possesses genes for a phycoerythrin protein but the sequence is degenerative relative to functional sequences for such phycobiliproteins (98). These organisms do not possess a gene for RpaC (61). Thus RpaC might require phycobilisomes to perform its function. Yet, there are low-light *P. marinus* ecotypes that have an *rpaC* gene but do not express phycobilisomes, though do express more functional phycobiliproteins than the high-light ecotype MED4 (98).

Thus in the context of an evolutionary propensity, one can correlate the disappearance of RpaC with the degeneration of phycobiliproteins and subsequent ecotype-shift toward high light. This provides evidence that RpaC regulates phycobilisomes and that evolutionary pressure exists to remove it under conditions where association of even remnant phycobiliproteins could enhance dangerous, excess energy production from PSII.

To my knowledge few state transition mutants have been found that are defective in energy transfer to PSI. This suggests that the phycobilisome-PSI structures are so evolutionarily robust and significant that perhaps numerous modifications to multiple genes would be required to remove such associations. However, one exception is the PsaK2 subunit of PS I which, when deleted, only impairs state transitions under high light (17). In contrast, removal of just one of a number of genes (*petM*, *ssr2998*, *rpaC*, *apcD*, *rpaC*, *apcE*) impairs or completely inhibits excitation energy transfer from phycobilisomes to PSII. Thus the association of phycobilisomes with PS II seems evolutionarily weak. That the association can be disrupted in numerous ways suggests that specific and complex interactions are involved and coordinated among at least six or more components. One may liken this to a form of an evolutionary fuse, that though easy to break, saves the electrical system from catching fire.

Mullineaux and coworkers have performed much research on state transitions, notably with FRAP (Fluorescence Recover After Photo-bleaching) analysis to assess the various contributions phycobilisome diffusion plays in state transitions. As noted previously, the organism he prefers (*Synechococcus* 7961) contains only CpcG1-type

phycobilisomes and thus only the 'mobile' phycobilisome. Thus many of their conclusions are understandable and not in contention with spill-over model arguments forwarded by experiments performed in *Synechocystis* 6803. Many of these findings are summed up in the Mullineaux et al. FRAP paper (61). They suggest that PS II complexes are immobile (61) during FRAP. If arranged in long fence-like complexes, as discussed above, one can imagine they are relatively immobile. In DesA knockout mutants, the membrane is made more fluid and diffusion of phycobilisomes was found to be 120 fold slower than in the wild type. Under such fluid conditions phycobilisomes preferred PSII, although this may not have been a functional energy transferring association and PS I fluorescence was higher. Joshua and Mullineaux (60) conclude that this is evidence that a membrane component is not involved in keeping phycobilisomes associated with the membrane during diffusion, and that the phycobilisomes remain on the membrane due to multiple weak interactions. According to the Sombrero Model proposed in this thesis (Section 4.3.4), membrane instability should inhibit the Ssr2998/FNR anchoring of the phycobilisomes and their association with PSII. I do not contest that phycobilisomes interact weakly with the membrane phospholipids head-groups and thereby maintain a degree of adhesion to the membrane. My model suggests that the cytochrome *bf* complex simply anchors the phycobilisomes long enough for them to associate with PSII. Such anchoring might be inhibited by membrane fluidity. Yet in the Mullineaux et al. study (60), PS II appears preferentially associated with phycobilisomes, suggesting the reverse is happening. However, I wonder about the redox state of the electron transport chain in such a mutant. One might expect, as seen in other 'leaky' mutants, such as Δ PetM, that

PS II seems to be the preferred site for phycobilisomes in over-oxidized mutants. The DesA mutants grows more slowly at room temperature and below compared to wild type and the *desA* mutation made PS II more susceptible to photoinhibition (60). This mutation results in slower PS II turn-over and over oxidation of the plastoquinone pool, resulting in migration of phycobilisomes to PSII. I suggest that over-oxidation of the PQ pool in the DesA mutant has locked the PSII-phycobilisome complexes into the fence-like structures illustrated in Figure 18 A. This could be responsible for the inhibited phycobilisome diffusion observed by Joshua and Mullineaux (60).

4.4 –Future Research Directions and Concluding Statements

4.4.1 – ROS Production and Future Studies.

4.4.1.1 – ROS production from the cytochrome *bf* complex. I have shown that ROS production in *Synechocystis* 6803 is enhanced by NQNO and decreased by TDS (Figure 8). Additionally, TDS lowered the ROS production caused by NQNO. This indicates these ROS are produced from the cytochrome *bf* complex and extends the previous findings from Horn (27). The site of ROS production within the cytochrome *bf* complex may be difficult to decipher with the instruments in our lab. However, electron paramagnetic resonance (EPR) spectroscopy of inhibited cytochrome *bf* complexes might shed light on where free-radicals are produced in this complex. Whether the Q_p or the Q_n site is the primary contributor of ROS may be difficult to conclude because ROS from one site cannot be deciphered from ROS produced by the opposing site by means of H₂DCFDA. Additional studies into this topic would include isolating cytochrome *bf*

complexes and conducting studies using XTT (3'-[1-[(phenylamino)carbonyl]-3,4-tetrazolium]bis(4-methoxy-6-nitro) and H₂DCFA (2',7'-dichlorodihydrofluorescein diacetate) and other biochemical assays capable of deciphering and quantifying the types of ROS produced from the complex. These experiments should be repeated in the isolated cytochrome *bf* complexes with TDS and/or NQNO to compare the ROS production-type profiles. The effect of FCCP concentration on ROS production from the cytochrome *bf* complex should also be investigated *in vivo*, because state transition and other experiments (48) indicate that FCCP drastically alters the midpoint potential of the c_n heme at the Q_n site of the cytochrome *bf* complex. If ROS were found to correlate with FCCP concentration, this would provide significant evidence that the source of ROS production from the cytochrome *bf* complex is the Q_n site.

4.4.1.2 – ROS production and redox-sensing and signaling. Experiments attempting to identify a link between ROS and state transitions were problematic. Addition of an exogenous ROS (hydrogen peroxide) did significantly alter state transitions (data not shown), but only at extremely high concentrations (0.3% Hydrogen peroxide). However, the dose dependent effects of exogenously added hydrogen peroxide might allow even small but continuous amounts of ROS to have a large impact as indicated by the PetB-R214H mutant of *Synechococcus* PCC 7002. In conclusion, ROS are harmful for cells, with an over-reduced plastoquinone pool leading to higher rates of ROS production and ultimately cellular damage and slowed growth. State transitions should help mitigate this impact, but we have no evidence at present that ROS may signal state transitions.

Continued research in this area would be needed to understand whether state transitions are indeed diminished due to ROS production. In 77K spectra of cells treated with inhibitors, it seems that state transition may have been perturbed relative to the separate fluorescence yields of PS I and PSII. Thus Further study in this area should include 77K spectra of cells exposed to these lower concentrations of ROS over time to determine if changes in PS I relative to PS II occur over-time or if one photosystem is selectively reduced in content in response to increased concentrations of ROS. My prediction is that PS I fluorescence would increase dramatically and that the stress response itself would elicit an IsiA-mediated increase in PS I. Moreover, I also believe this is the mechanism by which higher amounts of ROS and longer exposure to ROS induce reduction of PS II fluorescence and increased phycobilisome association with PSI.

4.4.2 – Continuing the Story of Redox Regulation by The Cytochrome *bf* Complex.

Studies here using NQNO and TDS have provided evidence that the cytochrome *bf* complex is specifically and significantly involved in light-induced state transitions. The impact of NQNO on inhibition of the dark-to-light state transition when added in the dark has held up under scrutiny from two different methods at room temperature and 77K. These studies (Figures 10-15) suggested two separate redox sensing/signaling mechanisms, therefore a fresh view of the mobile-phycobilisome and spill-over models of state transitions were required to explain the differential effects of NQNO and TDS on state transitions. The resulting model, Sombrero Grande and Cytochrome *bf* Conformation Model is, I believe, a step in the direction toward understanding the

transient and elusive nature of state transitions, but its components must be tested. The model can be used to make predictions that should be tested based on the primary components and proposed functions of the model.

4.4.2.1 – Model Predictions regarding the Cytochrome *bf* complex. The Sombrero Grande and Cytochrome *bf* Conformation Model (hereafter The Model) predicts that if the embedded chlorophyll of the cytochrome *bf* complex is removed, Light Transitions (dark to light transitions) would be significantly inhibited. However, cytochrome *f* re-reduction rates should be unaffected under anaerobic conditions. In addition, Light Poise Transitions should be absent.

4.4.2.2 – Model Predictions regarding Photosystem II. The Model suggests that fence-like arrangements of PSII-phytycobilisomes-cytochrome *bf* complex supercomplexes (PBS-superstructure) are determined by the relative redox state of the cytochrome *bf* complex low potential domain, and that association of protein components such as PetP and PetM facilitate in the arrangement of the PBS-superstructure. If NQNO were added to dark-adapted cultures of cyanobacteria, and these were then exposed to light and compared with un-treated cultures, the Model predicts that the fence-like PBS-superstructure would look more similar to that in dark-adapted cultures than light-adapted cultures. Furthermore, The Model predicts that Δ PetM and Δ PetP mutant cultures would display PBS-superstructures more similar to those in dark-adapted cultures than those in light adapted cultures. In regards to Light Poise Transitions (the dynamic building and collapse of PBS-superstructures), disorganization of the PBS-superstructure should be sensitive to FCCP treatments and associated changes in cytoplasmic pH.

4.4.2.3 – Model Predictions regarding Photosystem I. The Model suggests that Dark Poise Transitions and Dark Transitions (light to dark transitions) would occur much faster in the *Synechocystis* 6803 PsaL mutant, where PS I trimers cannot form (99). Likewise, there would be a smaller change in PS I fluorescence on Light Transition when compared to the wild-type because the phycobilisome to monomer ratio produces higher PS I fluorescence than phycobilisome to trimers due to the Sombrero Effect (Section 4.3.2).

4.4.2.4 – Model Predictions regarding Phycobilisomes. The Model suggests that ferredoxin NADP reductase (FNR) should have a docking site with the cytochrome *bf* complex and that NQNO or removal of PetP would induce a drop in NADPH concentrations in the cytosol because impairing FNR binding to cytochrome *bf* complex (PetP), or impairing the energy source for FNR reduction of NADP (NQNO blocking the Q_p site) should lower the amount of available NADPH. Additionally, attempts to isolate phycobilisome-cytochrome *bf* structures, e.g. by careful extraction in the presence of light, and analyze them by electron microscopy or mass spectrometry may yield interesting evidence of such interactions and the proteins involved.

References

1. Harper, M-E., Monemdjou, S., Ramsey, J., and Weindruch, R. (1998). Age-related increase in mitochondrial proton leak and decrease in ATP turnover reactions in mouse hepatocytes. *American Phys. Soc.* 275. 2, 197.
2. Wiseman, H. and Haliwell, B. (1996). Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem J.* 313, 17
3. Schriener, S., Linford, N., Martin, G., Treuting, P., Ogburn, C., Edmond, M., Coskum, P., Ladiges, W., Wolf, N., Van Remmen, H., Wallace, D., and Rabinovitch, P. (2005) Extension of Murine Life Span by Overexpression of Catalase Targeted to Mitochondria. *Science.* 308, 1909.
4. Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J., Rovio, A., Bruder, C., Bohlooly, M., Gidlo, S., Oldfors, A., Wibom, R., Tornell, J., Jacobs, H., and Larsson, N-G. (2004). Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature.* 429, 417.
5. Loeb, L., Wallace, D., and Martin, G. (2005) The mitochondrial theory of aging and its relationship to reactive oxygen species damage and somatic mtDNA mutations. *Proc. Natl. Acad. Sci.* 102, 52, 18769.
6. Shigenaga, M., Hagen, T., and Ames, B. (1994). Oxidative damage and mitochondrial decay in aging. *Proc. Natl. Acad. Sci.* 91, 10771.
7. Finkel, T., and Holbrook, N. (2000) Oxidants, oxidative stress and the biology of ageing. *Nature.* 408, 239.
8. Rao, P., Hayon, E. (1975) Redox potentials of free radicals. Superoxide and hydroperoxy radicals O₂⁻ and H₂O₂. *J. Phys. Chem.* 79, 397

9. Clelend, R., and Grace, S. (1999) Voltametric detection of superoxide production by photosystem II. *FEBS Letters*. 457, 348.
10. Zelko, I., Mariani, T., and Folz, R. (2002) Superoxide Dismutase Multigene Family: A comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radical Biology and Medicine*. 33, 3, 337.
11. Brown, M., Miller, F., Li, W., Ellingson, A., Mozena, J., Chatterjee, P., Engelhardt, J., Zwacka, R., Oberley, L., Fang, X., Spector, A., Weintraub, N. (1999) Overexpression of Human Catalase Inhibits Proliferation and Promotes Apoptosis in Vascular Smooth Muscle Cells. *Circulation Res*. 85, 524.
12. Li, S., Yan, T., Yang, J-Q., Oberley, T., and Oberley, L. (2000) The Role of Cellular Glutathione Peroxidase Redox Regulation in the Suppression of Tumor Cell Growth by Manganese Superoxide Dismutase. *Cancer Res*. 60, 3927.
13. Hart, S., Schlarb-Ridley, B., Bendall, D., and Howe, C. (2005) Terminal oxidases of cyanobacteria. *Biochemical Society Transactions*. 33, 832.
14. Darnell, J., Lodish, H., Baltimore, D. (1990) Photosynthesis. 617-638.
15. Campbell, D. (1998) Chlorophyll Fluorescence Analysis of Cyanobacterial Photosynthesis and Acclimation. *Microbiology and Molecular Biology Reviews*. 62, 3, 667.
16. Allen, J., (2004) Cytochrome b6f: structure for signaling and vectorial metabolism. *Trends Plant Sci*. 9, 130.
17. Hihara, Y., Sonoike, K., Kanehisa, M., and Ikeuchi, M. (2003) DNA microarray analysis of redox-responsive genes in the genome of the cyanobacterium *Synechocystis* sp. Strain PCC 6803. *J. Bact*. 185: 1719.

18. McConnell, M., Koop, R., Vasil'ev, S., and Bruce, D. (2002) Regulation of the Distribution of Chlorophyll and Phycobilin-Absorbed Excitation Energy in Cyanobacteria. A structure-Based Model for the Light State Transition. *Plant. Phys.* 130, 1201.
19. Foyer, C., and Noctor, G. (2003) Redox sensing and signaling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiologia Plantarum.* 119, 355.
20. Ježek, P., Žáčková, M., Růžička, M., Škobisová, E., Jabůrek, M. (2004) Mitochondrial Uncoupling Proteins – Facts and Fantasies. *Physiol. Res.* 53, 200.
21. Perelman, A., Uzan, A., Hacoheh, D., and Schwarz, R. (2003) Oxidative Stress in *Synechococcus* sp. Strain PCC 7942: Various Mechanisms for H₂O₂ Detoxification with Different Physiological Roles. *J. Bact.* 185, 3654.
22. Miller, A., Hunter, K., O'Leary, S., and Hart, L. (2000) The Photoreduction of H₂O₂ by *Synechococcus* sp. PCC 7942 and UTEX 6251. *Plant Phys.* 123, 625.
23. Zapata, J. Gue'ra, A., Esteban-Carrasco, A., Martı'n, M., and Sabater, B. (2005) Chloroplasts regulate leaf senescence: delayed senescence in transgenic ndhF-defective tobacco. *Cell Death and Differentiation.* 12, 1277.
24. Li, H., Singh, A., McIntyre, L., and Sherman, L. (2004) Differential Gene Expression in Response to Hydrogen Peroxide and Putative PerR Regulon of the *Synechocystis* sp. Strain PCC 6803. *J. Bact.* 186, 11, 3331.
25. Cadoret, J-C., Rousseau, B., Perewoska, I., Sicora, C., Cheregi, O., Vass, I., and Houmard, J. (2005) Cyclic Nucleotides, the Photosynthetic Apparatus and the Response to a UV-B Stress in the cyanobacterium *Synechocystis* 6803. *JBC*, 280, 40, 33935.

26. Cooley, J., and Vermaas, W. (2001) Succinate Dehydrogenase and Other Respiratory Pathways in Thylakoid Membranes of *Synechocystis* sp. Strain PCC 6803: Capacity Comparisons and Physiological Function. *J. Bact.* 183, 14, 4251.
27. Horn, D. (2004) Superoxide Production in the Cytochrome *bf* Complex of Photosynthesis. *Master's Thesis*, University of Wisconsin Oshkosh.
28. Nelson, N., and Ben-Shem, A. (2004) The Complex Architecture of Oxygenic Photosynthesis. *Nature Reviews Molecular Cell Biology*. 5, 1.
29. Crofts, A. (2004) The Q-cycle – a personal perspective. *Photosynthesis Research. Minireview*.
30. Li, N., Ragheb, K., Lawler, G., Sturgis, J., Rajwa, B., Melendez, J., and Robinson, P. (2003) Mitochondrial Complex I Inhibitor Rotenone Induces Apoptosis through Enhancing Mitochondrial Reactive Oxygen Species Production. *JBC*. Vol. 278, 8516.
31. Zhao, Z., Rothery, R., and Weiner, J. (1999) Stopped-flow studies of the binding of 2-n-heptyl-4-hydroxyquinoline-N-oxide to fumarate reductase of *Escherichia coli*. *Eur J Biochem*. 260, 50.
32. Nomura, C., Persson, S., Shen, G., Inoue-Sakamoto, K., and Bryant, D. (2006) Characterization of two cytochrome oxidase operons in the marine cyanobacterium *Synechococcus* sp. PCC 7002: Inactivation of *ctaDI* affects the PS I:PS II ratio. *Photosynthesis Res.* 87, 215.
33. Pils, D., and Schmetterer, G. (2001) Characterization of three bioenergetically active respiratory terminal oxidases in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *FEMS Microbiol Let.* 203, 217.
34. Nelson, M., Finazzi, G., Wang, Q., Middleton-Zarka, K., Whitmarsh, J., and Kallas,

- Toivo. (2005) Cytochrome b_6 Arginine 214 of *Synechococcus* cp. PCC 7002, a Key Residue for Quinone-reductase Site Function and Turnover of the Cytochrome bf Complex. *J. Biol. Chem.* Vol. 280, 11, 10395.
35. Kurisu, G., Zhang, H., Smith, J., Cramer, WA. (2003) Structure of the cytochrome b_6f complex of oxygenic photosynthesis: Tuning the cavity. *Science*. 302, 1009.
 36. Stroebel, D., Choquet, Y., Popot, J., Picot, D. (2003) An atypical haem in the cytochrome b_6f complex. *Nature*. 426, 413.
 37. Kallas, T., Spillerand, S., Malkin, R. (1988) Characterization of Two Operons Encoding the Cytochrome b_6 -f Complex of the Cyanobacterium *Nostoc* PCC 7906: Highly Conserved Sequences but Different Gene Organization than in Chloroplasts. *JBC*. 263, 14334.
 38. Widger, W., Cramer, W., Herrmann, R., and Trebst, A. (1984) Sequence homology and structural similarity between cytochrome b of mitochondrial complex III and the chloroplast bf complex: Position of the cytochrome b hemes in the membrane. *Proc. Natl. Acad. Sci.* 81, 674.
 39. Berthold, D., Schmidt, C., and Malkin, R. (1997) The deletion of *petG* in *Chlamydomonas reinhardtii* Disrupts the Cytochrome bf complex. *JBC*. 270. 49, 29293.
 40. Hamel, P., Olive, J., Pierre, Y., Wollman, F, De Vitry, C. (2000) A new subunit of cytochrome bf complex undergoes reversible phosphorylation upon state transition. *JBC*. 275, 17072.
 41. Barbagallo, R., Breyton, C., and Finazzi, G. (2000) Kinetic effects of the electrochemical proton gradient on plastoquinone reduction at the Q_i site of the cytochrome b_6f complex. *JBC*. 275, 26121.

42. Vener, A., Van Kan, P., Rich, P., Ohad, I., and Andersson, B. (1997) Plastoquinol at the quinol oxidation site of reduced cytochrome *bf* mediates signal transduction between light and protein phosphorylation: Thylakoid protein kinase deactivation by single-turnover flash. *Proc. Natl. Acad. Sci.* 94, 1585.
43. Vener, A., Van Kan, P., Gal, A., Ohad, I., and Andersson, B. (1995) Activation/Deactivation Cycle of Redox-controlled Thylakoid Protein Phosphorylation: Role of Plastoquinol Bound to The Reduced Cytochrome Bf Complex. *JBC.* 270, 25225.
44. Zhang, H., Huang, D., Cramer, W. (1999) Stoichiometrically Bound β -Carotene in the Cytochrome *b₆f* Complex of Oxygenic Photosynthesis Protects against Oxygen Damage. *JBC.* 274,1581.
45. Dashdorj, N., Zhang, H., Kim, H., Yan, J., Cramer, W., Savikhin, S. (2005) Unusual Optical Properties of the monomeric Chlorophyll a in the cytochrome bf complex of oxygenic photosynthesis. *International Society of Photosynthesis.* 88, 4178.
46. Wenk, S-O., Schneider, D., Boronowsky, U., Jäger, C., Klughammer, C., De Weerd, F., Van Roon, H., Vermaas, W., Dekker, J., Rögner, M. (2005) Functional implications of the pigments bound to a cyanobacterial cytochrome *bf* complex. *FEBS Jour.* 272, 582.
47. Kallas, T. (1994) The cytochrome *b₆f* complex. The Molecular Biology of Cyanobacteria. *Kluwer Academic Publishers.* Bryant DA. 259-317.
48. Alric, J., Pierre, Y., Picot, D., Lavergne, J., and Rappaport, F. (2005) Spectral and Redox characterization of the heme Ci of the cytochrome bf complex. . *Proc. Natl. Acad. Sci.* 102,44,15860.
49. Johnson, G. (2004) Cyclic electron transport in C3 plants: fact or artefact? *J.*

- Experimental Bot.* 56, 411, 407.
50. Munekage, Y., Hashimoto, M., Miyake, C., Tomizawa, K-I., Endo, T., Tasaka, M., and Shikanai, T. (2004) Cyclic electron flow around photosystem I is essential for photosynthesis. *Nature*, 429, 579.
 51. Munekage, Y., and Shikanai, T. (2005) Cyclic electron transport through photosystem I. *Plant Biotech.* 22, 361.
 52. Shikanai, T., Endo, T., Hashimoto, T., Yamada, Y., Asada, K., and Yokota, A. (1998) Directed disruption of the tobacco *ndhB* gene impairs cyclic electron flow around photosystem I. *Proc. Natl. Acad. Sci.* 95, 9705.
 53. Thomas, D., Thomas, J., Youderian, P., and Herbert, S. (2001) Photoinhibition and Light-Induced Cyclic Electron Transport in the *ndhB*- and *psaE*- Mutants of *Synechocystis* sp. PCC 6803. *Plant Cell Phys.* 42,8, 803.
 54. Endo, T., Shikanai, T., Sato, F., and Asada, K. (1998) NAD(P)H Dehydrogenase-Dependent, Antimycin A-Sensitive Electron Donation to Plastoquinone in Tobacco Chloroplasts. *Plant & Cell Phys.* 39, 11, 1226.
 55. Charlebois, D. and Mauzerall, D. (1999) Energy storage and optical cross-section of PS I in the cyanobacterium *Synechococcus* PCC 7002 and a *psaE*- mutant. *Photosynthesis Res.* 59, 1, 27.
 56. Hope, A., Birch, S., Matthews, D. (1987) Further Studies of Proton Translocations in Chloroplasts After Single-Turnover Flashes. IV. Effects of Cytochrome b/f Complex Inhibitors on Proton Uptake and Cytochrome b6 Turnover . *Australian Journal of Plant Physiology* 14, 4.

57. Ogawa, T. (1991) A gene homologous to the subunit-2 gene of NADH dehydrogenase is essential to inorganic carbon transport of *Synechocystis* PCC6803. *Proc. Natl. Acad. Sci.* 88, 4275.
58. Xu, D., Liu, X., Zhao, J., and Zhao, J. (2005) FesM, a Membrane Iron-Sulfur Protein, Is Required for Cyclic Electron Flow around Photosystem I and Photoheterotrophic Growth of the Cyanobacterium *Synechococcus* sp. PCC 7002. *Plant Phys.* 138, 1586.
59. Emlyn-Jones, D., Ashby, M., and Mullineaux, C. (1999) A gene required for the regulation of photosynthetic light harvesting in the cyanobacterium *Synechocystis* 6803. *Molec. Microbio.* 33, 5, 1050.
60. Joshua, S., and Mullineaux, C. (2004) Phycobilisome Diffusion Is Required for Light-State Transitions in Cyanobacteria. *Plant Phys.* 135, 2112.
61. Emlyn-Jones, D., and Mullineaux, C. (2004) State transitions: an example of acclimation to low-light stress. *J. Experimental Bio.* 56, 411, 389.
62. Finazzi, G. (2004) The central role of the green alga *Chlamydomonas reinhardtii* in revealing the mechanism of state transitions. *J. Experimental Botony.* 56, 383.
63. Finazzi, G., Zito, F., Barbagallo, R., and Wollman, F. (2001) Contrasted effects of inhibitors of cytochrome b6f complex on state transitions in *Chlamydomonas reinhardtii*: the role of Qo site occupancy in LHCII kinase activation. *JBC.* 276, 9770.
64. Galyna, I., Kufryk, and Vermaas, W. (2006) Sll1717 Affects the Redox State of the Plastoquinone Pool by Modulating Quinol Oxidase Activity in Thylakoids. *J. Bacterio.* 188, 1286.

65. Vallon, O., Bulte, L., Dainese, P., Olive, J., Bassi, R., and Wollman, F. (1991) Lateral redistribution of the cytochrome *bf* complexes along the thylakoid membranes upon state transition. *Proc. Natl. Acad. Sci.* 88, 8286.
66. Anderson, L., Rayner, M., Sweet, R., and Eiserling, F. (1983) Regulation of *Nostoc* sp. Phycobilisome Structure by Light and Temperature. *J. Bact.* 155,3,1407.
67. Yan, J., and Cramer, W. (2003) Functional insensitivity of the cytochrome b6f complex to structure changes in the hinge region of the Rieske iron-sulfur protein. *J. Biol. Chem.* 278, 20925-20933.
68. Tarpey, M., Wink, D., and Grisham, M. (2004) Methods for detection of reactive metabolites of oxygen and nitrogen: *in vitro* and *in vivo* considerations. *Am J Physiol Regul Integr Comp Physiol.* 286, 431.
69. Samuelson, G., Prezelin, B. (1985) Photosynthetic Electron Transport in Cell-Free Extracts of Diverse Phytoplankton. *J Phycol.* 21, 453.
70. Repine, J., Pfenninger, O., Talmage, D., Berger, E., and Pettijohn, D. (1981) Dimethyl sulfoxide prevents DNA nicking mediated by ionizing radiation or iron/hydrogen peroxide-generated hydroxyl radical. *Proc. Natl. Acad. Sci. USA*, 78, 1001.
71. Jones, R. and Whitmarsh J. (1987) Inhibition of electron transfer and the electrogenic reaction in the cytochrome *bf* complex by 2-*n*-nonyl-4-hydroxyquinoline *N*-oxide (NQNO) and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB). *Biochimica Biophysica Acta.* 933, 258.
72. Jones, R., and Whitmarsh, J. (1984) Origin of the electrogenic reaction in the chloroplast cytochrome *bf* complex. *Photobiochem. and Photobiophysics.* 9, 119.

73. Yamashita, E., Zhang, H., and Cramer, W. (2007) Structure of the Cytochrome *b6f* Complex: Quinone Analogue Inhibitors as Ligands of Heme *cn*. *J Mol. Biol.* 370, 39.
74. Benz, R. and Mclaughlin, S. (1983) The Molecular Mechanism of Action Of The Proton Ionophore Fccp (Carbonylcyanide P-Trifluoromethoxyphenylhydrazone). *J Biophys.* 41, 381.
75. He, Y-Y, and Häder, D. (2002) Reactive oxygen species and UV-B: effect on cyanobacteria. *Photochem. Photobiol. Sci.* 1, 729.
76. Invitrogen – Molecular Probes. (2006) Reactive Oxygen Species (ROS) Detection Reagents. *Company Product Publication*.
77. Wilson, A., Ajlani, G., Verbavatz, J-M., Vass, I., Kerfeld, C., and Kirilovsky, D. (2006) A Soluble Carotenoid Protein Involved in Phycobilisome-Related Energy Dissipation in Cyanobacteria. *The Plant Cell.* 18, 992.
78. Ivanov, A., Krol, M., Sveshnikov, D., Selstam, E., Sandström, S., Koochek, M., Park, Y., Vasil'ev, S., Bruce, D., O'quist, G., and Huner, N. (2006) Iron Deficiency in Cyanobacteria Causes Monomerization of Photosystem I Trimers and Reduces the Capacity for State Transitions and the Effective Absorption Cross Section of Photosystem I in Vivo. *Plant Physiology.* 141, 1436.
79. Kondo, K., Ochiai, Y., Katayama, M., and Ikeuchi, M. (2007) The Membrane-Associated CpcG2-Phycobilisome in *Synechocystis*: A New Photosystem I Antenna. *Plant Physiology.* 144, 1200.
80. Shen, G., Boussiba, S., and Vermaas, W. (1993) *Synechocystis* sp PCC 6803 and Phycobilisome Function Strains Lacking Photosystem I. 5, 1853.

81. Van Thor, J., Gruters, O., Matthijs, H., and Hellingwerf, K. (1999) Localization and function of ferredoxin:NADPF reductase bound to the phycobilisomes of *Synechocystis*. *EMBO*. Vol. 18, 4128.
82. Thomas, J-C, Ughy, B., Lagoutte, B., and Ajlani, G. (2006). A second isoform of the ferredoxin:NADP oxidoreductase generated by an in-frame initiation of translation. *Proc. Natl. Acad. Sci.* 103, 18368.
83. Li, H., Li, D., Yang, S., Xie, J., Zhao, J. (2006) The state transition mechanism—simply depending on light-on and -off in *Spirulina platensis*. *Biochemica et Biophysica Acta*. 1757, 1512.
84. De Lavalette, AdL., Finazzi, G., and Zito, F. (2008) *B6f*-Associated Chlorophyll: Structural and Dynamic Contribution to the Different Cytochrome Functions. *Biochemistry*, 47, 19.
85. Heimann, S., Ponamarev, M., and Cramer, W. (2000) Movement of the Rieske Iron-Sulfur Protein in the p-Side Bulk Aqueous Phase: Effect of Lumenal Viscosity on Redox Reactions of the Cytochrome b6f Complex. *Biochemistry*. 39, 2692.
86. Chain, R. and Malkin, R. (1995) Functional activities of monomeric and dimeric forms of the chloroplast cytochrome b6f complex. *Photosynthesis Res.* 46, 419.
87. Breyton, C., Tribet, C., Olive, J., Dubacqi, J-P, and Popot, J-L. (1996) Dimer to Monomer Conversion of the Cytochrome b6 f Complex :Causes And Consequences. *JBC*. 272, 21892.
88. Zhang, R., Li, H., Xie, J., and Zhao, J. (2007) Estimation of relative contribution of “mobile phycobilisome” and “energy spillover” in the light–dark induced state transition in *Spirulina platensis*. *Photosynthesis. Res.* Acquired from author.

89. Kondo, K., Ochiai, Y., Katayama, M., and Ikeuchi, M. (2007) The Membrane-Associated CpcG2-Phycobilisome in *Synechocystis*: A New Photosystem I Antenna. *Plant Physiology*. 144, 1200.
90. Schneider, D., Berry, S., Rich, P., Seidler, A., Rögner, M. (2001) A regulatory role of the PetM subunit in cyanobacterial cytochrome *bf* complex. *JBC*. 276,16780.
91. Volkmer, T., Schneider, D., Bernát, G., Kirchhoff, H., Wenk, S-O, and Rögner, M. (2007) Ssr2998 of *Synechocystis* sp PCC 6803 is involved in regulation of cyanobacterial electron transport and associated with the cytochrome *bf* complex. *JBC*. 282(6):3730-7.
92. Hihara, Y., Sonoike, K., and Ikeuchi, M. (1998) A Novel Gene, *pmgA*, Specifically Regulates Photosystem Stoichiometry in the Cyanobacterium *Synechocystis* Species PCC 6803 in Response to High Light. *Plant Phys*. 117. 1205.
93. Schneider, D., Berry, S., Seidler, A., and & Rögner, M. (1998) Evidence for a nonessential function of the small subunit PetM in the cytochrome b6f complex of *Synechocystis* PCC 6803. In *Photosynthesis, Mechanisms and Effects* (Garab G & Puztai J, eds) Vol. III, pp. 1545– 1548. Kluwer Academic Publishers, Dordrecht, the Netherlands.
94. Joshua, S., Mullineaux, C. (2005) The rpaC gene product regulates phycobilisome–photosystem II interaction in cyanobacteria. *Biochimica et Biophysica Acta*. 1709, 58.
95. Kuhl, H., Rogner, M., Van Breemen, J., and Boekema, E. (1999) Localization of cyanobacterial photosystem II donor-side subunits by electron microscopy and the supramolecular organization of photosystem II in the thylakoid membrane. *Eur. J. Biochem*. 266, 453.

96. Morschel, E. and Schatz, G. (1987) Correlation of photosystem-II complexes with exo-plasmatic freeze-fracture particles of thylakoids of the cyanobacterium *Synechococcus* sp. *Planta* 172, 145.
97. Sener, M., Park, S., Lu, D., Damjanovic, A., Ritz, T., Fromme, P., and Schulten, K. (2004) Excitation migration in trimeric cyanobacterial photosystem I. *J. Chem. Physics*. 120, 11183.
98. Ting, C., Rocap, G., King, J., and Chisholm, S. (2001) Phycobiliprotein genes of the marine photosynthetic prokaryote *Prochlorococcus*: evidence for rapid evolution of genetic heterogeneity. *Microbiology*, 147, 3171.
99. Schluchter, W., Shen, G., Zhao, J. and Bryant, D. (1996). Characterization of *psaI* and *psaL* Mutants of *Synechococcus* sp. Strain PCC 7002: A New Model for State Transitions in Cyanobacteria. *Photochemistry and Photobiology*, 64: 53–66.
100. Barber, J. (2002) P680: what is it and where is it? *Bioelectrochemistry*. 55, 135.
101. Rappaport, F., Guergova-Kuras, M., Nixon, P., Diner, B., and Lavergne, J. (2002) Kinetics and pathways of charge recombination in photosystem II. *Biochemistry*. Jul. 41: 8518-8527.
102. Ivanov, B., and Khorobrykh, S. (2003) Participation of photosynthetic electron transport in production and scavenging of reactive oxygen species. *Antioxidants and Redox Signaling*. 5, 43.
103. Takamiya, K., and Dutton, PL. (1979) Ubiquinone in *Rhodospseudomonas sphaeroides* Some thermodynamic properties. *Biochim Biophys Acta*. 546, 1.
104. Crofts, A., Wang, Z. (1989) How rapid are the internal reactions of the UQH₂:cyt c₂ oxidoreductase? *Photosynth Res*.22: 69-87.

105. T'sai, A., and Palmer, G. (1983). Potentiometric studies on yeast complex III. *Biochim Biophys Acta*. 722, 349.
106. Kramer, D., and Crofts, A. (1994) Re-examination of the properties of the *b* cytochromes of the thylakoid *bf* complex. *Biochim Biophys Acta*. 1184, 193.
107. Nitschke, W., Joliot, P., Liebl, U., Rutherford, A., Hauske, G., Muller, A., and Riedel, A. (1992) The pH dependence of the redox midpoint potential of the 2Fe2S cluster from the cytochrome *b6f* complex (the 'Rieske center'). *Biochim Biophys Acta*. 1102, 266.
108. Riedel, A., Rutherford, A., Hauska, G., Muller, A., and Nitschke, W. (1991) Chloroplast Rieske center EPR study on its special characteristics, relaxation and orientation properties. *JBC*. 239, 3017.
109. Hurt, E. and Hauska, G. (1982) Identification of polypeptides in the cytochrome *b6f* complexes from spinach chloroplasts with redox-center carrying components. *J Bioenerg Biomembr*. 14,405.
110. Levine, R., and Gorman, D. (1966) Photosynthetic electron transport chain of *Chlamydomonas reinhardtii*. Light-induced absorbance changes in chloroplast fragments of the wild type and mutant strains. *Plant Physiol*. 41, 1293.
111. Ramesh, V., Guergova-Kuras, M., Joliot, P., and Webber, A. (2002) Electron transfer from plastocyanin to the photosystem I reaction center in mutants with increased potential of primary donor in *Chlamydomonas reinhardtii*. *Biochemistry*. 41, 14652.
112. Munge, B., Das, S., Ilagan, R., Pendon, Z., Yang, J., Frank, H., and Rusling, J. (2003) Electron transfer reactions of redox cofactors in spinach photosystem I reaction center protein in lipid films on electrodes. *J Am Chem Soc*. 125, 12457.

113. Parrett, K., Mehari, T., Warren, P., and Golbeck, J. (1989) Purification and properties of the intact P-700 and Fx-containing photosystem I core protein. *Biochim Biophys Acta*. 97,324.
114. Evans, M., Reeves, S., and Cammack, R. (1974) Determination of the oxidation-reduction potential of the bound iron-sulphur proteins of the primary electron acceptor complex of photosystem I in spinach chloroplast. *FEBS Lett.* 49, 111.
115. Cammack, R., Rao, K., Barger, C., Hutson, K., Andrew, P., Rogers, R. (1977) Midpoint redox potential of plant and algal ferredoxin. *JBC*. 168, 205.
116. Kyte, J. and Doolittle, R. (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 157:105.