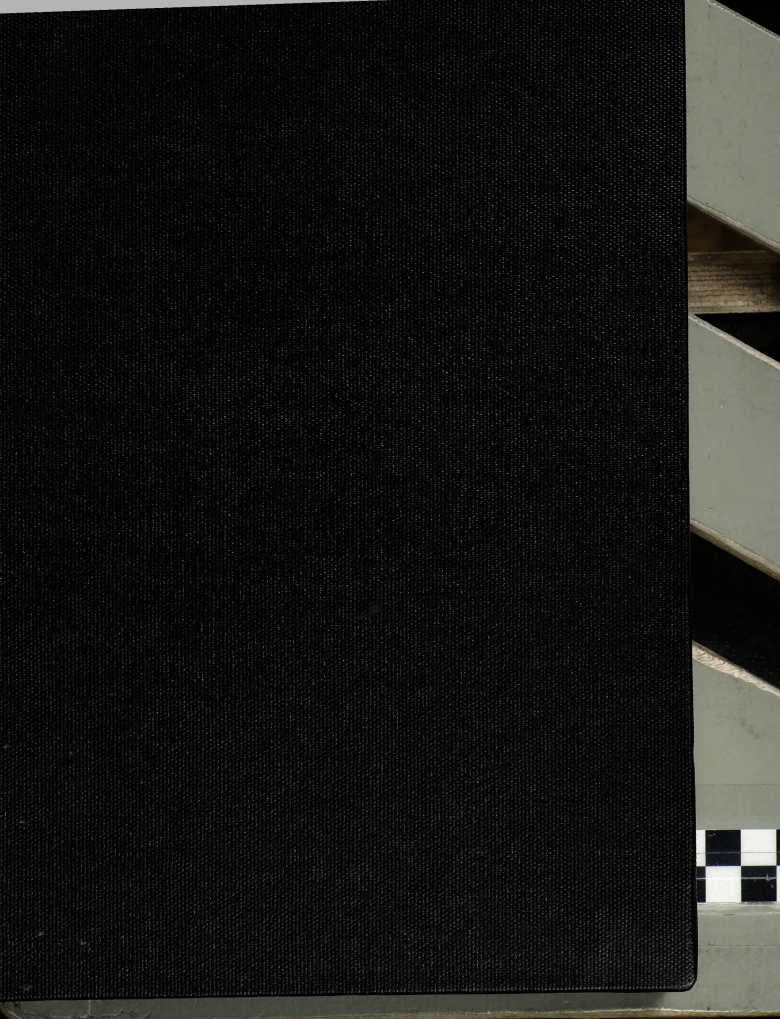

This is to certify that the

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Molecular analysis of the photosystem I reaction center in the cyanobacterium *Synechocystis* sp. PCC 6803

presented by

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By

Lawrence B. Smart

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

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1992

ABSTRACT

MOLECULAR ANALYSIS OF THE PHOTOSYSTEM I REACTION CENTER IN THE CYANOBACTERIUM *SYNECHOCYSTIS* SP. PCC 6803

By

Lawrence B. Smart

The focus of this study is the investigation of the biogenesis, structure, and function of the photosystem (PS) I reaction center in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 using molecular genetic techniques. The PSI reaction center is comprised of a heterodimer of homologous 82 - 83-Kda polypeptides, PsaA and PsaB, which bind all but two of the electron transfer components of PSI and are encoded by the genes *psaA* and *psaB*. The structure-function elements studied in this project were the [4Fe-4S] electron transfer component F_X, which is coordinated by four cysteine residues, two from PsaA and two from PsaB, and a leucine zipper motif in PsaA and PsaB, proposed to act in reaction center dimerization. *Synechocystis* 6803 is an excellent organism for the study of PSI, because it is readily transformable, has an active homologous recombination system, has a photosynthetic apparatus much like that of plants, and may be grown heterotrophically. The *psaA-psaB* operon was isolated and its DNA sequence determined. Transcripts from *psaA-psaB* accumulate in dark-grown cells to a level similar to that in light-grown cells. Transcripts from *psbA* also accumulate in dark-grown cells, but to levels three- to four-fold lower than in light-grown cells. Both the *psaA* and

psaB genes were inactivated by interposon mutagenesis and selection under light-activated heterotrophic growth (LAHG) conditions, indicating that the PSI reaction center is dispensable for heterotrophic growth of *Synechocystis* 6803. Genetic inactivation of PSI was performed in strains to be used for PSII mutagenesis in order to facilitate the purification and analysis of PSII. Site-directed amino acid substitutions were incorporated into PsaB, changing a proposed cysteine ligand of F_X and changing leucines in a leucine zipper motif. Analysis of these mutants indicates that F_X is an important structural as well as functional component of PSI, but that the leucine zipper may not play a major role in reaction center dimerization.

ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to those people who have shared their precious time, knowledge, experience, and enthusiasm with me in the process of completing this project. I am pleased to have had the opportunity to exchange ideas, techniques, and home-brews with my collaborators at the University of Nebraska, Patrick Warren and Dr. John Golbeck. I am indebted to Dr. Neil Bowlby for teaching me much of what I know about photosynthesis, EPR spectroscopy, and ball bearings. I wish to thank Kurt Stepnitz for his excellent and extensive photographic work on my behalf. I have been very fortunate to have had the enthusiastic support and encouragement of my advisory committee, Dr. Barbara Sears, Dr. Michael Thomashow, and Dr. C. Peter Wolk. Equally supportive have been my co-workers in the McIntosh lab, especially Dr. John Fitchen, Dr. Carrie Hiser, Dr. Idah Sithole, Dr. Shawn Anderson, and Dr. David Rhoads. I must give a great deal of credit for the success of this project and for the good start of my scientific career to Dr. Lee McIntosh, who has given unending support and guidance. Finally, I would like to thank my wife, the future Dr. Christine Durbahn Smart and my parents, Robert and Marjorie Smart, for their loving understanding, tolerance, and encouragement.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
cab	chlorophyll *a/b* binding
chl	chlorophyll
DCBQ	2,6-dichloro-*p*-benzoquinone
DCMU	N_1-(dichlorophenyl)-N_3-dimethylurea
ENDOR	electron-nuclear double resonance
ESEEM	electron spin echo envelope modulation
ESR	electron spin resonance
EPR	electron paramagnetic resonance
EXAFS	electron X-ray absorption fine structure
F_A	[4Fe-4S] center A
F_B	[4Fe-4S] center B
F_X	[4Fe-4S] center X
Gm	gentamycin
kb	kilobases or kilobase pairs
kDa	kiloDalton
Km	kanamycin
LAHG	light-activated heterotrophic growth
LHC	light harvesting complex
mT	milliTesla
NADP	nicotinamide adenine dinucleotide phosphate
PAGE	polyacrylamide gel electrophoresis
PCC	Pasteur Culture Collection
PCR	polymerase chain reaction
PEG	polyethylene glycol
PSI	photosystem I
PSII	photosystem II
SDS	sodium dodecyl sulfate
Sp	spectinomycin
Sm	streptomycin
TES	*N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid
Tris	tris(hydroxymethyl)aminomethane
WT-MIXO	mixotrophically-grown wild type
WT-LAHG	LAHG-grown wild type

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CHAPTER 1

INTRODUCTION

PHOTOSYNTHESIS

Photosynthesis, the process used by plants, algae, and some bacteria to convert light energy into chemical energy, dramatically affects the Earth and its atmosphere. This series of chemical reactions, catalyzed by dozens of polypeptides, is responsible for generating the O_2 that we breathe and for fixing CO_2 into organic compounds. Thus, to ensure our survival in an ever-changing environment, it is vital for us to gain a complete understanding of the mechanisms and regulation of these reactions and the proteins that catalyze them.

Photosynthesis can be classified into oxygenic and non-oxygenic. Plants, algae, and cyanobacteria perform oxygenic photosynthesis, evolving O_2 from the light-driven oxidation of water and transferring electrons through a linear series of carriers, eventually yielding reducing equivalents, in addition to performing cyclic photosynthesis. Non-oxygenic photosynthesis is characterized by cyclic electron flow through a single photo-reaction center, driving ATP synthesis, but not the generation of reducing equivalents. Non-oxygenic photosynthesis is

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performed by species of bacteria and has been best characterized in the purple bacteria *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis* (Deisenhofer *et al.,* 1985; Allen *et al.,* 1987). Further discussion of the photosynthetic proteins in the purple and green bacteria and the heliobacteria will be limited to their use as models for some of the proteins involved in oxygenic photosynthesis and the evolutionary significance of that similarity.

As alluded to earlier, oxygenic photosynthesis involves the light-driven flow of electrons from water to reduce nicotinamide adenine dinucleotide phosphate ($NADP^+$) to NADPH, which may be used as reducing power for many anabolic reactions. This process is accomplished in plants, algae, and cyanobacteria by three thylakoid membrane-bound, multi-subunit enzyme complexes: photosystem II (PSII), the cytochrome b_6f complex, and photosystem I (PSI). As electrons are passed through certain carriers, a proton gradient is generated across the thylakoid membrane. This gradient is released by the flow of protons through the ATP synthetase (also called the coupling factor), driving ATP synthesis. ATP and NADPH act as substrates for carbon fixation and sugar synthesis, amino acid synthesis, fatty acid synthesis, or other anabolic reactions. In plants and algae, the thylakoids and many of the enzymes involved in biosynthetic processes are compartmentalized to the chloroplasts.

The basic requirements for the light-dependent photosynthetic reactions are: light capture, charge separation, and charge stabilization. To accomplish efficient energy use, the charge separation created at the initial photochemical

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event must be quickly stabilized by a series of oxidation-reduction reactions between spatially separate electron carriers. The initial step is the capture of energy from light by antennae pigments, primarily chlorophylls. This energy is then transferred to special, reaction center chlorophyll, which in PSII is termed P_{680} (Rutherford, 1988). The energy captured by P_{680} generates an excited electron, which is then passed to the primary acceptor, a phaeophytin, then to a bound plastoquinone, Q_A (Rutherford, 1988). Finally, electrons are passed to a second plastoquinone called Q_B, which when doubly reduced to plastoquinol, exchanges with a quinone from a pool in the membrane (Rutherford, 1988). P_{680}^+ is a strong oxidant and is reduced by electrons donated by tyrosine radicals in the D1 and D2 polypeptides, termed Y_Z and Y_D (Debus *et al.,* 1988a; Debus *et al.,* 1988b). Y_Z^+ and Y_D^+ are reduced by Mn atoms in the oxygen evolving complex. The tetrad of Mn atoms accumulates oxidizing equivalents in a linear series of oxidation or S-states, finally oxidizing water and releasing oxygen in a single event (Kok *et al.,* 1970; Forbush *et al.,* 1971).

The plastoquinol form of Q_B reduces the cytochrome b_6/f complex. The mechanism of electron transport through this multisubunit complex is poorly understood, but it catalyzes the net import of two protons for every two electrons it transports (O'Keefe, 1988). The cyt b_6/f complex is shared between photosynthesis and respiration in the cyanobacteria (Sandmann *et al.,* 1984). The cytochrome b_6/f complex also accomplishes the transition from the two electron carrier Q_B to the single electron carrier plastocyanin, which may be replaced by cytochrome c_{553} in cyanobacteria (Sandmann, 1986). Plastocyanin,

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like Q_B, is a diffusible carrier that can bridge the distinct protein complexes in the thylakoid. Plastocyanin (or sometimes cytochrome c_{553} in cyanobacteria) donates electrons to the reaction center chlorophyll pair in PSI, P_{700}.

PSI captures the second photon of light needed to complete reduction of $NADP^+$ and transfers the energy to P_{700}, creating the strongest reductant in a biological system (≈-1.2 V) (Vos and van Gorkom, 1988). P_{700} passes an energized electron to the primary electron acceptor A_0, which donates electrons to A_1, which then reduces F_X. Finally, F_X reduces F_A and/or F_B, which then donate electrons to ferredoxin. In cyanobacteria, flavodoxin can substitute for ferredoxin under conditions of iron-limitation (Ho and Krogmann, 1982). Ferredoxin then reduces $NADP^+$ to NADPH; a reaction catalyzed by the ferredoxin:$NADP^+$ oxidoreductase (Ho and Krogmann, 1982). The detailed nature of the electron carriers in PSI will be discussed below. PSI is also capable of catalyzing cyclic electron flow, in which electrons are returned to the cytochrome complex to cycle back to P_{700} (O'Keefe, 1988). This process does not generate reducing power (NADPH), but does cause formation of a proton gradient for ATP synthesis.

PHOTOSYSTEM I

PSI reaction center

The PSI complex is a conglomeration of many protein subunits, both membrane-bound and extrinsic, which coordinate several different cofactors

involved in electron transport. Recently, detailed reviews of PSI structure, function, and gene organization have been published and provide the reader a valuable compilation of the current state of understanding of PSI (Bryant, 1987; Golbeck, 1989; Golbeck, 1992; Golbeck and Bryant, 1991; Mathis and Rutherford, 1987). The nomenclature used to describe the PSI subunits and the genes that encode them is as follows: genes are named as in other bacterial systems, with three lower case letters describing the function of the encoded protein, then an upper case letter to describe a particular gene in a group; the entire name is italicized (Hallick, 1989). In the case of PSI, the first two letters, *ps*, signify photosynthesis, the next, *a*, signifies PSI (as opposed to *b*, signifying PSII), and the final letter currently ranges from *A* to *L*, designating a particular gene. However, to avoid the confusion that has arisen from the naming of the protein subunits by their order of electrophoretic migration, which varies between organisms (Golbeck and Bryant, 1991), protein nomenclature will differ from that of Hallick (1989). The protein name is the same as the gene name, but is not italicized, and the first and last letters are capitalized. The list of subunits presented here represents a complete list as we know it, but more subunits may be discovered in the future as techniques to detect them become more refined.

The reaction center of PSI consists of a heterodimer of homologous 82 - 83-kDa (kiloDalton) polypeptides, named PsaA and PsaB (Cantrell and Bryant, 1987; Fish *et al.,* 1985). The amino acid sequences deduced from the *psaA* and *psaB* genes are predicted to form 11 membrane-spanning α-helices (Cantrell

and Bryant, 1987; Fish *et al.,* 1985). There are approximately 80 histidine residues in PsaA and PsaB combined, which may bind many of the ≈100 chlorophyll molecules associated with the reaction center, including P_{700} (Golbeck and Bryant, 1991). In plants and algae, antennae chlorophylls are also associated with chlorophyll *a/b*-binding (cab) proteins in the light-harvesting complex (LHC) (Golbeck, 1992). However, in cyanobacteria all of the chlorophyll associated with PSI appears to be bound to the reaction center proteins, as there are no LHC proteins (Ho and Krogmann, 1982).

The PsaA and PsaB proteins bind the electron transfer components P_{700}, A_0, A_1, and F_X. The reaction center chlorophyll, P_{700}, is the site of the primary photochemical charge separation in PSI. It is named by the wavelength at the peak of the oxidized-minus-reduced difference spectrum (Kok, 1956). Optical and electron paramagnetic resonance (EPR) spectroscopy indicate that P_{700} is a chlorophyll *a*-dimer (Kok, 1957; Norris *et al.,* 1974), analogous to the bacteriochlorophyll special pair P_{870} in the bacterial reaction center (Deisenhofer *et al.,* 1985). Recent data suggest that the ground state of P_{700} is a dimer, but that the P_{700}^+ cation is localized to one of the two chlorophyll molecules (Golbeck and Bryant, 1991; Ikegami and Itoh, 1988). Upon absorbing a photon of light, P_{700} is raised to an excited state, P_{700}^*, then passes an electron to A_0 and forms the cation P_{700}^+. P_{700}^+ is detectable by EPR spectroscopy, giving a signal centered at g=2.0025, with a linewidth of 0.8 - 1.0 milliTesla (mT), termed signal I (Norris *et al.,* 1971). P_{700}^+ is reduced by plastocyanin or in cyanobacteria, by cytochrome c_{553} (Sandmann, 1986).

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The primary electron acceptor, A_0, has been best characterized by time-resolved optical spectroscopy, in which the flash-induced absorption changes of P_{700} are measured under highly reducing conditions. Using this technique, the back reaction from A_0^- to P_{700}^+ has been determined to be approximately 35 ns (Mathis *et al.,* 1988). The flash-induced difference spectrum of A_0^- - A_0 indicates that this component is a chlorophyll *a* molecule (Mathis *et al.,* 1988; Golbeck and Bryant, 1991). Photo-accumulation experiments, in which continuous illumination is used under highly reducing conditions to obtain a state with P_{700} A_0^-, also suggests that A_0 is a chlorophyll *a* monomer present in a ratio of 0.94 A_0 /P_{700} (Mansfield and Evans, 1985).

The identity of the second electron acceptor, A_1, is controversial. A_1 has been characterized by photoaccumulation studies, using optical and EPR spectroscopy, and also by time-resolved optical spectroscopy. The absorption difference spectra and EPR spectra obtained in photoaccumulation studies are characteristic of some quinone species (Mansfield and Evans, 1985). Also, two molecules of phylloquinone (vitamin K_1) can be extracted from the PSI reaction center (Schoeder and Lockau, 1986). However, conflicting data exist concerning the role of phylloquinone in electron transport. Ether extraction to remove phylloquinone also blocks electron flow past A_0 at room temperature as measured by time-resolved optical spectroscopy (Mansfield *et al.,* 1987). However, under cryogenic temperatures, electron transfer to F_A is possible, suggesting that a low-temperature bypass of A_1 occurs (Setif *et al.,* 1987). In contrast, inactivation of phylloquinone by ultraviolet (UV) radiation does not

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eliminate the A_1 EPR signal (Ziegler *et al.,* 1987) or affect room temperature electron flow to the terminal electron acceptors (Palace *et al.,* 1987). Likewise, specific deuteration of quinone in a methionine auxotroph of *Anabaena* did not affect the photoaccumulated A_1 EPR signal (Barry *et al.,* 1988), casting doubt on the source of this EPR signal. The presence of phylloquinone in PSI, the analogy with the quinones in PSII, and data from optical spectroscopy after extraction of quinones are strong evidence for a role of phylloquinone in PSI electron transport.

The final electron transfer component bound to the reaction center proteins is F_X. This component was first detected by EPR spectroscopy of PSI preparations at low temperature (Evans *et al.,* 1976; Malkin and Bearden, 1978). After extraction with mild detergent, the 30 ms backreaction of F_A/F_B^- to P_{700}^+ is replaced by a 1.2 ms backreaction with a difference spectrum characteristic of an iron-sulfur center (Golbeck and Cornelius, 1986). Determination of acid-labile sulfur content of the PSI complex indicated that there were 12 mol of sulfur per mol of P_{700} (Golbeck *et al.,* 1988). Since F_A and F_B were known to be [4Fe-4S] centers (Wynn and Malkin, 1988a), the remaining iron and sulfur form either a [4Fe-4S] center or 2 [2Fe-2S] centers. Initial attempts to characterize F_X were complicated by interfering signals from the iron-sulfur centers F_A and F_B. Once a particle was resolved lacking F_A and F_B , but with F_X intact (Parrett *et al.,* 1989), confirmation of F_X as a [4Fe-4S] center was accomplished by extended X-ray absorption fine structure (EXAFS) analysis (McDermott *et al.,* 1989) and by Mössbauer spectroscopy (Petrouleas

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et al., 1989). Also the acid-labile sulfur content of this preparation is reduced from 12 mol / mol P_{700} to approximately 4 mol / mol P_{700} (Parrett *et al.,* 1989). The EPR spectrum of reduced F_X is only detected at low temperature and has *g* values of 2.08, 1.88, and 1.78 (Warden and Golbeck, 1986). These resonances are shifted slightly from those expected for a [4Fe-4S] center and the features are somewhat broader than expected (Golbeck and Bryant, 1991). An iron-sulfur center with the especially low redox potential of F_X (-670 mV) (Parrett *et al.,* 1989) is assumed to be coordinated by four cysteine residues (Golbeck and Cornelius, 1986). Analysis of conserved cysteines in the protein sequences of PsaA and PsaB shows that there are only three in PsaA and two in PsaB (Golbeck and Bryant, 1991), which indicates that F_X may be coordinated by both the PsaA and PsaB proteins and that the stoichiometry is of one PsaA polypeptide and one PsaB polypeptide per P_{700} (Golbeck, 1987b; Scheller *et al.,* 1989a). This stoichiometry has been confirmed by radiolabelling experiments (Bruce and Malkin, 1988b; Bruce and Malkin, 1988a). The role of F_X as an intermediate between A_1 and F_A/F_B is still not fully resolved, mainly due to the difficulty associated with its detection (Golbeck and Bryant, 1991).

A comparison of the five conserved cysteine residues found in PsaA and PsaB reveals that four of them lie in a motif conserved in both PsaA and PsaB containing two of the cysteines each (Golbeck and Bryant, 1991; Cantrell and Bryant, 1987). This 12 residue sequence is: Phe-Pro-Cys-Asp-Gly-Pro-Gly-Arg-Gly-Gly-Thr-Cys. These four cysteines, two in PsaA and two in PsaB have been proposed to act as the ligands to the [4Fe-4S] F_X (Golbeck and Cornelius,

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1986). Immediately adjacent to this motif in both PsaA and PsaB are heptad repeats of conserved leucine residues that are predicted to lie in α-helices (Fish *et al.,* 1985; Cantrell and Bryant, 1987) and have been proposed to form a leucine zipper (Webber and Malkin, 1990; Kössel *et al.,* 1990). Leucine zippers were discovered initially in some DNA-binding regulatory proteins, which function as dimers (Turner and Tjian, 1989; Landschulz *et al.,* 1989; Gentz *et al.,* 1989). It was shown with the proteins Fos and Jun and with the protein C/EBP that the leucine zippers were responsible for dimerization of the proteins, which positions basic regions in each protein in proximity to one another, forming a DNA-binding domain (Landschulz *et al.,* 1988; Landschulz *et al.,* 1989; Gentz *et al.,* 1989). Proteins with these characteristics have been termed bZIP proteins (Pu and Struhl, 1991). It has been shown by site-directed mutagenesis that it is the specific interaction of the leucines that is responsible for dimerization (Hu *et al.,* 1990; Landschulz *et al.,* 1989; Gentz *et al.,* 1989), but that other residues in the helices also play a role in specificity (O'Shea *et al.,* 1992). Synthetic peptides forming the GCN4 leucine zipper region have been crystallized and the structure resolved, revealing that the helices form a coiled-coil structure (O'Shea *et al.,* 1991). It has been proposed that the putative leucine zipper in PSI acts in the dimerization of the reaction center proteins, thus putting the cysteine ligands to F_X in position to coordinate the iron-sulfur center, analogous to the DNA-binding domain of bZIP proteins (Webber and Malkin, 1990; Kössel *et al.,* 1990).

Peripheral PSI polypeptides

The 9-kDa PsaC protein carries the terminal electron acceptors F_A and F_B. In time-resolved optical studies, F_A/F_B^- backreact with P_{700}^+ in 30 ms at room temperature, but this charge separation is irreversible at low temperature (Parrett *et al.,* 1989). F_A and F_B have been shown to be [4Fe-4S] centers by EPR spectroscopy (Cammack and Evans, 1975), EXAFS studies (McDermott *et al.,* 1988), Mössbauer spectroscopy (Evans *et al.,* 1979), and ^{19}F-NMR core extrusion studies (Golbeck *et al.,* 1987a). The EPR spectrum of F_A^- has characteristic *g* values of 2.05, 1.94, and 1.86, while the spectrum of F_B^- has *g* values of 2.07, 1.92, and 1.89 (Malkin and Bearden, 1978). When both centers are reduced either chemically with dithionite or by illumination during freezing, their high-field and low-field resonances merge, yielding a spectrum with *g* values of 2.05, 1.94, 1.92, and 1.89 (Evans *et al.,* 1974).

The *psaC* gene has been inactivated in a cyanobacterium, *Anabaena variabilis* sp. ATCC 29413 (Mannan *et al.,* 1991), and in the alga, *Chlamydomonas reinhardtii* (Takahashi *et al.,* 1991). In *Anabaena variabilis* 29413, the loss of PsaC prevented autotrophic growth and caused the cells to be light-sensitive, but did not significantly alter stable assembly of the PSI reaction center (Mannan *et al.,* 1991). However, in *Chlamydomonas*, inactivation of *psaC* prevented stable accumulation of the PSI reaction center proteins and of seven additional peripheral PSI polypeptides (Takahashi *et al.,* 1991).

The sequence of the PsaC protein includes an amino acid motif

characteristic of bacterial-type ferredoxins, which also bind 2 [4Fe-4S] centers (Høj *et al.,* 1987; Oh-oka *et al.,* 1988). This motif, Cys-X-X-Cys-X-X-Cys-X-X-X-Cys-Pro, is duplicated in the protein sequence and includes the eight cysteine ligands to the two iron-sulfur centers (Høj *et al.,* 1987; Oh-oka *et al.,* 1988). The three-dimensional structures of several bacterial-type ferredoxins are known, revealing that the protein backbone folds back on itself and that the fourth cysteine in each motif coordinates the opposite iron-sulfur center than the other three cysteines in the motif (Adman *et al.,* 1973; Stout *et al.,* 1988; Fukuyama *et al.,* 1988). An *in vitro* reconstitution system was developed using purified reaction center protein from *Synechococcus* sp. PCC 6301 (PCC, Pasteur Culture Collection) and PsaC and PsaD purified from strains of *Escherichia coli* that were expressing, respectively, the *psaC* gene from *Synechococcus* sp. PCC 7002 and the *psaD* gene from *Nostoc* sp. PCC 8009 (Zhao *et al.,* 1990). This system has been used to study *E. coli*-expressed PsaC protein with site-directed changes of some of the proposed cysteine ligands to F_A and F_B (Zhao *et al.,* 1992). By changing the second cysteine in each motif to aspartate, as can occur naturally in some ferredoxins (Conover *et al.,* 1990; George *et al.,* 1989), an inactive [3Fe-4S] center was formed, without affecting the formation of the other [4Fe-4S] center (Zhao *et al.,* 1992). The remaining functional [4Fe-4S] center in the mutant proteins behaved similarly to wild type. It was thus deduced that the amino-terminal motif binds F_B and the carboxyl-terminal motif binds F_A (Zhao *et al.,* 1992).

The PsaD polypeptide has a mass of 15 - 16 kDa in cyanobacteria and

of 18 kDa in plants (Chitnis *et al.,* 1989a; Münch *et al.,* 1988). Cross-linking has shown that it plays a role in the docking of the diffusible [2Fe-2S] ferredoxin to the PSI core (Zilber and Malkin, 1988; Zanetti and Merati, 1987). Another role for PsaD was discovered using the *in vitro* reconstitution system described earlier. Although PsaC was able to bind to the reaction center and restore electron transfer to F_A and F_B, the addition of PsaD made the resulting complex more resistant to detergent and allowed wild-type levels of reduction of F_A and F_B (Li *et al.,* 1991). Thus PsaD also appears to play a role in the proper binding of PsaC to the reaction center. The *psaD* gene has been inactivated in *Synechocystis* sp. PCC 6803, resulting in reduced growth rate under autotrophic conditions, but wild-type growth rate under photoheterotrophic conditions (in the presence of glucose as a carbon source and N_1-(dichlorophenyl)-N_3-dimethylurea [DCMU]) (Chitnis *et al.,* 1989a). This supports the idea that PsaD plays a role in facilitating electron transfer to ferredoxin, while not affecting cyclic photosynthesis.

The PsaE protein has a mass of 8 - 10-kDa and appears to be located on the stromal side of PSI (Franzén *et al.,* 1989b). This position was implied by cross-linking of PsaE to PsaD, which also cross-links to ferredoxin (Oh-oka *et al.,* 1989). This association with PsaD suggests that PsaE may play a role in the association of PsaC and PsaD to the reaction center. The *psaE* gene has been inactivated in *Synechocystis* 6803 and in *Synechococcus* sp. PCC 7002, but the phenotype was essentially wild-type, indicating that PsaE is dispensable for PSI function (Chitnis *et al.,* 1989b; Bryant *et al.,* 1990). The PsaF protein has a

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mass of approximately 17 kDa and has several positively charged domains which may serve in its proposed role of facilitating binding of the negatively charged plastocyanin and/or cytochrome c_{553} to the reaction center (Steppuhn *et al.,* 1988; Franzén *et al.,* 1989b). The PsaF protein has been cross-linked to plastocyanin in spinach preparations (Wynn and Malkin, 1988b). However, when the *psaF* gene was inactivated in *Synechocystis* 6803, the cells grew autotrophically at wild-type rates, indicating that this protein is dispensable for PSI function (Chitnis *et al.,* 1991). The PsaG protein has a mass of approximately 10 kDa and, like PsaD, PsaE, and PsaF, does not appear to be membrane bound (Steppuhn *et al.,* 1988; Franzén *et al.,* 1989a). The deduced amino acid sequence of the *psaG* gene from barley shows high identity to that from spinach and is weakly homologous to PsaK from *Chlamydomonas* (Okkels *et al.,* 1992). The authors suggest that either *psaG* or *psaK* arose by a duplication of the other, and that they occupy symmetrical positions in PSI (Okkels *et al.,* 1992). The PsaH protein has a mass of approximately 10 - 11 kDa, is polar, and appears to be located on the stromal side of PSI (Steppuhn *et al.,* 1989; Franzén *et al.,* 1989a). PsaH does not appear to exist in cyanobacteria (Koike *et al.,* 1989; Rhiel and Bryant, 1988). This information together with a similar pattern of *psaH* gene regulation as the *cab* genes, suggests that PsaH may play a role in facilitating the association of LHC I with PSI (Steppuhn *et al.,* 1989).

Improved techniques of gel electrophoresis have allowed the recent discovery of three very small polypeptides associated with PSI (Koike *et al.,*

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1989). The PsaI and PsaJ proteins both have masses of approximately 4 - 5 kDa, are quite hydrophobic, and are predicted to form a α helix (Møller *et al.*, 1990; Ikeuchi *et al.*, 1991). The PsaI sequence has some identity to helix E of PsbD, inspiring a proposal that it is involved in quinone binding (Møller *et al.*, 1990), but there is no direct evidence that either PsaI or PsaJ is associated with any cofactors. The PsaK protein has a mass of approximately 8 kDa, is quite hydrophobic, and is predicted to form a single membrane-spanning helix (Franzén *et al.*, 1989a). No role for PsaK has been definitively assigned thus far. The most recent PSI protein to be discovered is the 18-kDa PsaL protein from barley (Okkels *et al.*, 1991). PsaL is very hydrophobic and is predicted to form two membrane-spanning α helices (Okkels *et al.*, 1991). This protein may not have been discovered in other organisms, because it comigrates with PsaE (Okkels *et al.*, 1991).

PSI gene organization

In eucaryotes, the genes that encode the PSI polypeptides are located in both the nucleus and the plastid. The genes encoded in the plastome are: *psaA*, *psaB*, *psaC*, *psaI*, and *psaJ*, the remaining genes *psaD*, *psaE*, *psaF*, *psaG*, *psaH*, *psaK*, and *psaL* are encoded in the nucleus. Many of these genes have been characterized in cyanobacteria, as well, with the notable exception of *psaH*, which appears to be absent from cyanobacteria (Rhiel and Bryant, 1988; Koike *et al.*, 1989). The following is a brief description of the structure of each gene and a summary of the organisms in which particular genes have been

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The *psaA* and *psaB* genes have been cloned and sequenced from tobacco (Shinozaki *et al.,* 1986), liverwort (Ohyama *et al.,* 1986), rice (Hiratsuka *et al.,* 1989), maize (Fish *et al.,* 1985), pea (Lehmbeck *et al.,* 1986), spinach (Kirsch *et al.,* 1986), *Euglena gracilis* (Cushman *et al.,* 1987), *Chlamydomonas reinhardtii* (Kück *et al.,* 1987), *Synechococcus* sp. PCC 7002 (Cantrell and Bryant, 1987), and *Synechococcus vulcanus* (Shimizu *et al.,* 1992). In plants and cyanobacteria, the *psaA* and *psaB* genes are arranged in an operon, with *psaA* transcribed first. In the green algae, the *psaA* gene has introns; in *Chlamydomonas reinhardtii psaA* is organized in three exons (Kück *et al.,* 1987), while in *Euglena gracilis*, the *psaA* gene is split into four exons (Cushman *et al.,* 1987). In *Chlamydomonas* these exons are encoded on opposite strands, and the transcripts are joined by *trans*-splicing (Kück *et al.,* 1987; Choquet *et al.,* 1988; Goldschmidt-Clermont *et al.,* 1990). The *psaA* and *psaB* genes characterized so far are all highly similar to each other, in the range of 80% and greater similarity (Golbeck and Bryant, 1991). The *psaA* sequence is also homologous to *psaB*, exhibiting sequence identity of approximately 40% (Golbeck and Bryant, 1991). This suggests that one of the two reaction center genes arose by a duplication of the other.

The *psaC* gene has been cloned and sequenced from tobacco (Shinozaki *et al.,* 1986; Hayashida *et al.,* 1987), rice (Hiratsuka *et al.,* 1989), liverwort (Ohyama *et al.,* 1986), maize (Schantz and Bogorad, 1988), pea (Dunn and Gray, 1988), wheat (Dunn and Gray, 1988), spinach (Steppuhn *et al.,* 1989),

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Chlamydomonas reinhardtii (Takahashi *et al.,* 1991), *Anabaena* sp. PCC 7120 (Mulligan and Jackman, 1992), *Anabaena variabilis* sp. ATCC 29413 (ATCC, American Type Culture Collection) (Mannan and Pakrasi, 1992), *Synechococcus vulcanus* (Shimizu *et al.,* 1990), *Nostoc* sp. PCC 8009 (Bryant *et al.,* 1990), *Synechococcus* sp. PCC 7002 (Bryant *et al.,* 1990), and two copies from *Synechocystis* sp. PCC 6803 (Anderson and McIntosh, 1991b; Steinmüller, 1992). In chloroplasts and some cyanobacteria, the *psaC* gene is flanked by the genes *ndhD* and *ndhE*, and is cotranscribed with *ndhD* (Schantz and Bogorad, 1988; Anderson and McIntosh, 1991b; Hayashida *et al.,* 1987). The *psaC* genes characterized thus far are all highly similar to one another (Golbeck and Bryant, 1991).

The *psaD* gene has been cloned and sequenced from the following cyanobacteria: *Nostoc* sp. PCC 8009 (Bryant *et al.,* 1990), *Synechococcus* sp. PCC 6301 (Wynn *et al.,* 1989), and *Synechocystis* sp. PCC 6803 (Chitnis *et al.,* 1989a). cDNA clones of the *psaD* gene have been isolated and sequenced from spinach (Münch *et al.,* 1988), tomato (Hoffman *et al.,* 1988), and tobacco (Yamamoto *et al.,* 1991). The plant sequences are over 80% identical to one another, while the cyanobacterial sequences are over 60% similar to each other (Golbeck and Bryant, 1991). The cyanobacterial sequences are approximately 55% similar to those from plants (Golbeck and Bryant, 1991).

Clones of *psaE* have been isolated and sequenced from spinach (Münch *et al.,* 1988), barley (Okkels *et al.,* 1988), *Chlamydomonas reinhardtii* (Franzén *et al.,* 1989b), *Nostoc* sp. PCC 8009 (Bryant *et al.,* 1990), *Synechococcus* sp.

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PCC 6301 (Bryant *et al.,* 1990), *Synechococcus* sp. PCC 7002 (Bryant *et al.,* 1990), and *Synechocystis* sp. PCC 6803 (Chitnis *et al.,* 1989b). The cyanobacterial genes are much shorter than those from plants and are approximately 73% identical to one another (Golbeck and Bryant, 1991). The high degree of similarity suggests some structural or functional constraints that prevent divergence.

The *psaF* gene has been cloned and sequenced from spinach (Steppuhn *et al.,* 1988), *Chlamydomonas reinhardtii* (Franzén *et al.,* 1989b), and *Synechocystis* sp. PCC 6803 (Chitnis *et al.,* 1991). The deduced amino acid sequence from the *Synechocystis* sp. PCC 6803 gene is approximately 50% identical to the deduced amino acid sequences from spinach or *Chlamydomonas* (Chitnis *et al.,* 1991). The *psaG* and *psaH* genes have been cloned and sequenced from spinach (Steppuhn *et al.,* 1988; Steppuhn *et al.,* 1989), barley (Okkels *et al.,* 1992; Okkels *et al.,* 1989) and *Chlamydomonas reinhardtii* (Franzén *et al.,* 1989a). The *psaI* gene has been cloned and sequenced from the chloroplast genomes of tobacco (Shinozaki *et al.,* 1986), liverwort (Ohyama *et al.,* 1986), rice (Hiratsuka *et al.,* 1989), barley (Scheller *et al.,* 1989b), pea (Nagano *et al.,* 1991) and wheat (Ogihara, Terachi, and Sasahuma, unpublished data). The *psaJ* gene has only been cloned and sequenced from tobacco (Shinozaki *et al.,* 1986), rice (Hiratsuka *et al.,* 1989), and liverwort (Ohyama *et al.,* 1986). The *psaK* gene has been cloned and sequenced from *Chlamydomonas reinhardtii* (Franzén *et al.,* 1989a), and *psaL* has been cloned and sequenced from barley (Okkels *et al.,* 1991).

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GENETIC MANIPULATION OF PSI

Procaryotic organisms

Molecular biology offers the researcher the ability to alter an element of a biological system in order to learn more about that element in the natural system. This approach is ideal for studying the roles of particular components of the photosynthetic apparatus. Certain organisms are especially well-suited for these types of experiments and have been used to great advantage in the study of photosynthesis. The following is a summary of the organisms used today for molecular genetic studies of oxygenic photosynthesis.

The cyanobacteria represent excellent organisms for the study of photosynthesis. Two strains in particular have been used most frequently and to best advantage, *Synechocystis* sp. PCC 6803 and *Anabaena variabilis* sp. ATCC 29413. Occasionally, other strains have been used, such as *Synechococcus* sp. PCC 7002 or *Synechococcus* sp. PCC 7942. *Synechocystis* 6803 is a unicellular cyanobacterium that is naturally competent, and thus is readily transformable (Grigorieva and Shestakov, 1982). It has a genome size comparable to *E. coli* and expresses bacterial drug resistance genes effectively (Williams, 1988). This strain has an active homologous recombination system, making gene replacement by double recombination a straightforward technique (Williams, 1988). There does not appear to be an active restriction or modification system in *Synechocystis* 6803 that would digest transforming DNA (Williams, 1988). Of great importance is that this strain may be grown

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photoheterotrophically (with DCMU and glucose) (Williams, 1988) or heterotrophically, under light-activated heterotrophic growth conditions (LAHG), complete darkness except 5 min of light every 24 h and in the presence of glucose (Anderson and McIntosh, 1991a). Growth under these conditions eliminates the selective advantage conferred by the expression of wild-type photosynthesis genes, allowing for complete segregation of mutations. *Synechocystis* 6803 has approximately 8 - 10 copies of its chromosome per cell (Williams, 1988); thus in order to obtain a homozygous strain, segregation is crucial. Techniques for the purification of both PSII and PSI from *Synechocystis* 6803 have been developed (Rögner *et al.,* 1990; Noren *et al.,* 1991; Burnap *et al.,* 1989). *Synechocystis* 6803 has been used to great advantage in the study of PSII structure and function relationships (Noren and Barry, 1992; Debus *et al.,* 1988b; Debus *et al.,* 1988a; Nixon and Diner, 1992; Vermaas *et al.,* 1988; Pakrasi *et al.,* 1988), PSII biogenesis (Jansson *et al.,* 1987; Nilsson *et al.,* 1990), and PSI biogenesis (Chitnis *et al.,* 1989b; Chitnis *et al.,* 1989a; Chitnis *et al.,* 1991).

Another cyanobacterium that has been used for the genetic manipulation of PSI is *Anabaena variabilis* sp. ATCC 29413. This filamentous cyanobacterium can grow heterotrophically, using fructose as a carbon source (Wolk and Shaffer, 1976). Plasmids for the conjugal transfer of DNA into *Anabaena variabilis* 29413 have been engineered (Elhai and Wolk, 1988; Murry and Wolk, 1991) and a method using a conditionally-lethal gene for the selection of double recombinants was developed (Cai and Wolk, 1990). The

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primary integration event that occurs after conjugation is single recombination, which is undesirable for site-directed mutagenesis or gene replacement experiments (Elhai and Wolk, 1988). By using the *sacB* gene, cells that have integrated conjugated DNA by single recombination are killed when grown on sucrose (Cai and Wolk, 1990). This strain has been used to inactivate the PSI genes *psaC* (Mannan *et al.,* 1991) and *psaB* (Toelge *et al.,* 1991).

Eucaryotic organisms

Although cyanobacteria are excellent organisms for studying oxygenic photosynthesis, they lack the additional elements of regulation and complexity associated with the interaction between the nucleus and plastids in plants and algae. The development of a technique for the transformation of chloroplasts has prompted some researchers to use molecular genetics to study photosynthesis in a species of green algae, *Chlamydomonas reinhardtii*, and in a higher plant, tobacco. *Chlamydomonas* is a unicellular alga with a single, relatively large chloroplast. The photosynthetic apparatus has been studied extensively in this organism, both at the protein level and the genetic level (Chua *et al.,* 1975; Rochaix and Erickson, 1988). The *Chlamydomonas* chloroplast may be transformed using the biolistic particle-bombardment technique (Boynton *et al.,* 1988; Newman *et al.,* 1991; Boynton *et al.,* 1990), and cells may be grown heterotrophically on acetate (Harris, 1989). Using a gene conferring resistance to spectinomycin/streptomycin for selection, the *psaC* gene has been inactivated in *Chlamydomonas* (Takahashi *et al.,* 1991). In

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addition, a *psaB* mutant has been restored to photosynthetic competence, by transforming with a wild-type *psaB* gene (Bingham *et al.*, 1991).

Genetic manipulation of photosynthesis in higher plants is also now possible, using the biolistic technique to transform tobacco (*Nicotiana tabacum*) chloroplasts (Svab *et al.*, 1990; Staub and Maliga, 1992). In tissue culture, these plants may be grown on glucose, allowing complete segregation of inactivation mutations of *psbA* and *rbcL*, using mutations in the 16S rRNA to confer resistance to spectinomycin (Svab and Maliga, 1991). Although the genetic manipulation of PSI genes in tobacco has not yet been attempted, the potential certainly exists for successful utilization of this organism in the study of PSI.

FOCUS OF THIS STUDY

The body of the work presented in this thesis addresses the development and utilization of the cyanobacterium *Synechocystis* sp. PCC 6803 as a system for the study of PSI structure/function relationships and biogenesis. The main thrust of the project was genetic manipulation of the PSI reaction center genes, in order to gain insight into the structural framework needed for proper assembly and function of the PSI reaction center. The initial stage of the project was the isolation and characterization of the *psaA-psaB* operon from *Synechocystis* sp. PCC 6803. This included analysis of *psaA-psaB* mRNA accumulation in cells grown under LAHG conditions, which were to be used for the selection of mutants in PSI. To ensure that the *psaA* and *psaB* genes are dispensable for the growth of *Synechocystis* 6803 under LAHG conditions and

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to study PSI biogenesis, both genes were inactivated by interposon mutagenesis. Since PSII assembled normally in cells with mutations that inactivated *psaA* or *psaB*, strains were engineered which have genetic inactivation mutations in *psaA* and could be used as recipients for PSII site-directed mutations. The lack of PSI in strains with directed mutations in either *psbA* or *psbD* allows for simple and effective purification of PSII in order to analyze the effects of those mutations. The final stage of the study addressed the role of the [4Fe-4S] center F_X and of the array of leucines in the reaction center using the technique of site-directed mutagenesis. A system for the rapid recovery of site-directed mutants in the *psaA* gene was developed and utilized to change a potential ligand to the iron-sulfur cluster F_X and to introduce mutations into the leucine zipper motif. Analysis of these mutants gives us insight into the role of F_X and of the leucine zipper in the biogenesis of the PSI reaction center. In addition I was able to study the participation of F_X in electron transport through the reaction center.

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CHAPTER 2

Expression of photosynthesis genes in the cyanobacterium *Synechocystis* sp. PCC 6803: *psaA-psaB* and *psbA* transcripts accumulate in dark-grown cells

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INTRODUCTION

The study of oxygenic photosynthesis has been expedited by the use of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 as a model system. The photosynthetic apparatus in cyanobacteria is highly homologous to that from higher plants, including subunit composition, functional components, and primary sequence (Ho and Krogmann, 1982). *Synechocystis* 6803 is very amenable to molecular genetic manipulation. It is naturally competent, is readily transformable, has an active homologous recombination mechanism, expresses bacterial drug resistance genes, and can be grown photoheterotrophically (Williams, 1988) or heterotrophically (Anderson and McIntosh, 1991). Considerable progress has been made in the study of PSII in *Synechocystis* 6803, including cloning and mutagenesis of the genes for the D1

and D2 core polypeptides, *psbA* (present in three copies) and *psbD* (present in two copies) (Debus *et al.,* 1988; Jansson *et al.,* 1987). In addition, mutagenesis of two peripheral proteins of PSI has been accomplished in *Synechocystis* 6803 (Chitnis *et al.,* 1989a; Chitnis *et al.,* 1989b). The recent discovery of heterotrophic growth conditions for *Synechocystis* 6803, which negate the selective advantage of wild-type PSI, make it an excellent system for molecular analysis of the core of PSI (Anderson and McIntosh, 1991). *Synechocystis* 6803 grows in the dark if supplied with glucose and 5 min of light every 24 h (Anderson and McIntosh, 1991). Anderson and McIntosh have termed this light-activated heterotrophic growth (LAHG) (Anderson and McIntosh, 1991).

The biochemistry of PSI from plants and cyanobacteria has been extensively characterized (Bengis and Nelson, 1977; Lundell *et al.,* 1985; Wynn *et al.,* 1989). Electron transfer components have been defined by various spectroscopic techniques and have been shown to be associated with three polypeptides, the two P_{700}-apoproteins (PsaA and PsaB) and the 9 kDa iron-sulfur (Fe-S) protein (PsaC) (Golbeck, 1989). PsaA and PsaB bind P_{700}, A_0, A_1, and F_X, while F_A and F_B are bound to PsaC (Golbeck *et al.,* 1988; Høj *et al.,* 1987). PsaA and PsaB also bind approximately 100 chlorophyll molecules for each P_{700} (Golbeck, 1989). The electron transfer component F_X is most likely a [4Fe-4S] center bound by four cysteine residues, two in PsaA and two in PsaB (Golbeck and Bryant, 1991). Immediately adjacent to those conserved cysteines in both PsaA and PsaB are series of conserved leucines, spaced seven residues apart, in regions predicted to form α-helices. It has been proposed that these

helices may interact to form a leucine zipper (Kössel *et al.*, 1990; Webber and Malkin, 1990).

The *psaA* and *psaB* genes have been cloned and sequenced from many different organisms, including: maize (Fish *et al.*, 1985), spinach (Kirsch *et al.*, 1986), pea (Lehmbeck *et al.*, 1986), tobacco (Shinozaki *et al.*, 1986), rice (Hiratsuka *et al.*, 1989), *Marchantia polymorpha* (Ohyama *et al.*, 1986), *Euglena gracilis* (Cushman *et al.*, 1987), *Chlamydomonas reinhardtii* (Kück *et al.*, 1987), and *Synechococcus* sp. PCC 7002 (Cantrell and Bryant, 1987). In plants and algae, these genes are located in the plastid genome. The *psaA* genes cloned from algae contain introns, and in *C. reinhardtii* the exons are joined by *trans*-splicing (Choquet *et al.*, 1988; Goldschmidt-Clermont *et al.*, 1990). In plants and *Synechococcus* 7002, the genes are arranged in tandem and have been shown to be co-transcribed (Cantrell and Bryant, 1987; Chen *et al.*, 1990; Meng *et al.*, 1988; Rodermel and Bogorad, 1985). All the *psaA* and *psaB* genes sequenced so far show a very high degree of conservation (Cantrell and Bryant, 1987).

The expression of photosynthesis genes in the process of plastid differentiation and chloroplast maturation has been the subject of intense research and has revealed the significant role of post-transcriptional regulation (for a recent review, see (Mullet, 1988)). The expression of photosynthesis genes has also been studied in cyanobacteria. Lönneborg *et al.* (Lönneborg *et al.*, 1988) showed there is greater accumulation of *psbA* transcript under high-light conditions relative to low-light conditions in *Synechococcus* sp. PCC

6301. Greater accumulation of *psbA* and *psbD* mRNA in high-light relative to low-light was also seen in *Synechocystis* 6803 (Mohamed and Jansson, 1989). However, no hybridization to mRNA from *psbA*, *psbD*, or *rbcL* (encoding the large subunit of ribulose-bisphosphate carboxylase/oxygenase [Rubisco]) could be detected from *Synechocystis* 6803 grown in the dark without glucose (Mohamed and Jansson, 1989). In *Synechocystis* 6803, copies 2 and 3 of *psbA* are nearly identical, while copy 1 is more divergent (Metz *et al.,* 1990; Osiewacz and McIntosh, 1987). By mutagenesis of two of the three copies of *psbA*, Mohamed and Jansson (1989) demonstrated that copy 1 is not expressed, while copies 2 and 3 are expressed in *Synechocystis* 6803. *Synechococcus* sp. PCC 7942 has three copies of *psbA* as well. However, copy 1, which encodes form I of the D1 protein, is expressed at much higher levels than copies 2 or 3, which encode form II of D1 (Bustos *et al.,* 1990; Schaefer and Golden, 1989b; Schaefer and Golden, 1989a). In *Synechococcus* 7942, the accumulation of mRNA from copies 2 and 3 decreases with decreasing light intensity, while the accumulation of mRNA from copy 1 increases with decreasing light intensity (Bustos *et al.,* 1990; Schaefer and Golden, 1989b).

This paper describes the cloning and sequencing of the *psaA* and *psaB* genes from *Synechocystis* sp. PCC 6803, and their use, together with other photosynthesis genes, as probes in the analysis of photosynthesis gene expression. Quantification of transcript accumulation for *psaA-psaB* and *psbA* in light-grown cells, cells put in total darkness with glucose, and LAHG cells will be presented.

MATERIALS AND METHODS

Materials

All chemicals and reagents used were of high quality and were obtained from Sigma Chemical Co. (St.Louis, MO), Boehringer Mannheim Biochemicals (Indianapolis, IN), or Research Organics (Cleveland, OH). Restriction and modification enzymes were purchased from New England Biolabs (Beverly, MA) or Gibco BRL (Gaithersburg, MD). Nitrocellulose was obtained from Schleicher and Schuell (Keene, NH). [α-^{32}P]dATP was obtained from Amersham (Arlington Heights, IL).

Strain and growth conditions

A glucose-tolerant (Williams, 1988), dark-growth-adapted (Anderson and McIntosh, 1991) strain of *Synechocystis* sp. PCC 6803 was grown in BG-11 medium supplemented with 5 mM TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid) (pH 8.0) as previously described (Jansson *et al.,* 1987). Glucose was added to a final concentration of 5 mM. Cell growth was measured by absorbance at 730 nm (OD_{730}). Light-grown cells received 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of continuous white light. LAHG conditions were: total darkness except for 5 min of white light (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) every 24 h as previously described (Anderson and McIntosh, 1991). For dark incubations, cells were grown to early exponential phase (OD_{730}=0.2-0.5), then wrapped in aluminum foil and put in a light-tight box in a darkroom accessed by a

darkened hallway for 24, 30, or 48 h. Cells grown without glucose were incubated for 48 h only in complete darkness as described (Mohamed and Jansson, 1989).

Cloning

All nucleic acid manipulations were performed using standard techniques (Sambrook *et al.,* 1989), except where otherwise noted. All DNA fragments used as hybridization probes (see Table 2.1) were purified by agarose gel electrophoresis and were random primer-labelled with ^{32}P (Feinberg and Vogelstein, 1983). Genomic DNA isolated from *Synechocystis* 6803 was purified on cesium chloride gradients as described (Williams, 1988). Conditions for hybridization with the *Synechococcus* 7002 probes were: 1X Denhardt's solution (0.2 mg/ml Ficoll, 0.2 mg/ml polyvinylpyrrolidone, 0.2 mg/ml bovine serum albumin), 3X SSC (a solution of 3 M NaCl and 0.3 M sodium citrate constitutes 20X SSC), 35% v/v formamide, 0.1% w/v sodium dodecyl sulfate (SDS), 40 mg/l salmon sperm DNA, 37°C. Hybridization conditions for *Synechocystis* 6803 probes were the same except 50% v/v formamide was used. Blots were washed with 6X SSC; two times at 25°C, then once at 37°C. A mini-library was constructed by ligating size-fractionated *Synechocystis* 6803 *Kpn* I fragments purified from an agarose gel into pUC119 (Vieira and Messing, 1987). *Escherichia coli* HB101 (Boyer and Roulland-Dussoix, 1969) was transformed with the ligation mixture and ampicillin resistant colonies were screened by colony hybridization (Grunstein and Hogness, 1975). A λEMBL3

library of *Synechocystis* 6803 DNA was previously described (Jansson *et al.*, 1987).

Sequencing and sequence analysis

DNA sequence was determined from either single-stranded or double-stranded templates generated from pUC118 or pUC119 (Vieira and Messing, 1987) by the chain-termination method (Sanger *et al.*, 1977) using Sequenase version 2 (U.S. Biochemical, Cleveland, OH) as described by the manufacturer. Nested deletions were generated using exonuclease III and mung bean nuclease (Henikoff, 1984). Oligonucleotides for use as sequencing primers were synthesized using an Applied Biosystems DNA synthesizer. These primers were used to sequence those regions not recovered in the series of exonuclease III deletions. The polymerase chain reaction (PCR) was performed using genomic *Synechocystis* 6803 DNA, two of the aforementioned oligonucleotides, and AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, CT), as described by the manufacturer. The amplified fragment was purified, digested, and subcloned into pUC118 for sequencing. DNA sequence analysis was performed using Editbase (courtesy of N. Neilson, Purdue University), MCF and Amphi (A. R. Crofts, Univ. of Illinois) and the programs of the University of Wisconsin Genetics Computer Group.

RNA isolation and northern blots

RNA was isolated from cells in late exponential phase (OD_{730}=0.7-0.9) using a previously described technique (Golden *et al.*, 1987), except that immediately after harvest, the cells were frozen at -70°C in BG-11, then thawed. Great care was taken to perform manipulations in complete darkness or very dim light until the cells were exposed to chloroform. RNA was denatured, fractionated on agarose/formaldehyde gels, and transferred to nitrocellulose. Molecular sizes were estimated by comparison to an ethidium bromide-stained lane of a 9.5-0.24 kb RNA ladder (Gibco-BRL). Northern blots were hybridized (1X Denhardt's solution, 3X SSC, 30 mM Tris pH 8.0, 0.1 mM EDTA, 50% v/v formamide, 0.1% w/v SDS, 40 mg/l salmon sperm DNA, 37°C) with ^{32}P random primer-labelled probes (see Table 2.1), washed with 6X SSC, then quantified using a Betascope machine (Betagen, Waltham, MA) and/or exposed to x-ray film. The blots were then stripped using boiling water with 0.2% w/v SDS (three times, 10 min each), verified to be non-radioactive, and reprobed.

RESULTS

Cloning

Southern blots of genomic *Synechocystis* 6803 DNA digested with *Kpn* I were probed with the plasmid pAQPR80 (the kind gift of D.A. Bryant), which contains the *psaA* and *psaB* genes from *Synechococcus* sp. PCC 7002 (Cantrell

Table 2.1. DNA probes used for screening, Southern, and northern hybridizations.

Source[a]	Genes encoded	Fragment
7002	*psaA,psaB*	pAQPR80 7.8 kb *Eco*R I-*Bgl* II-pUC9[b]
7002	85% of *psaA*	1.9 kb *Hin*d III (from pAQPR80)
7002	60% of *psaB*	1.4 kb *Hin*d III (from pAQPR80)
7002	5' end of *psaA*	0.5 kb *Eco*R I-*Hin*d III (from pAQPR80)
6803	80% of *psaA*	1.8 kb *Kpn* I (from pLS18)
6803	half of *psaB*	0.9 kb *Kpn* I (from pLS19)
6803	3' half of *psaB*	1.2 kb *Hin*d III (from pLS15)
6803	3' half of *psbA2*	0.5 kb *Kpn* I-*Hinc* II (from pKW1266[c])
6803	3' half of *psbD2*	0.6 kb *Kpn* I-*Sma* I (from pRD655[d])
6803	3' half of *rbcL*	0.7 kb *Bam*H I (from pSF2.6[e])
6803	*psaD*	0.4 kb *Bst*E II-*Bgl* I (from pPR8[f])
6803	3' flanking *psaB*	1.0 kb *Eco*R I-*Kpn* I (from pLS31)
7942	16S rRNA	2.0 kb *Xba* I (from pAN4[g])

[a] Organisms are: 7002, *Synechococcus* sp. PCC 7002; 6803, *Synechocystis* sp. PCC 6803; 7942, *Synechococcus* sp. PCC 7942

[b] (Cantrell and Bryant, 1987) kind gift of D.A. Bryant, Dept. of Molecular and Cell Biology, Pennsylvania State University, University Park, PA

[c] (Jansson *et al.,* 1987)

[d] (Debus *et al.,* 1988)

[e] T. Reich and L. McIntosh (unpublished)

[f] (Reilly *et al.,* 1988) kind gift of N. Nelson, Roche Institute, Nutley, NJ

[g] (Tomioka *et al.,* 1981) kind gift of S.S. Golden, Dept. of Biology, Texas A & M University, College Station, TX

and Bryant, 1987). Four hybridizing fragments of approximately 1.8, 1.7, 1.4, and 0.9 kilobase pairs (kb) were detected (data not shown). The sum length of these fragments corresponds to the expected length of the *psaA-psaB* operon (Cantrell and Bryant, 1987). *Kpn* I-digested genomic *Synechocystis* 6803 DNA was fractionated on an agarose gel, and the fragments corresponding in size to the hybridizing bands were purified and ligated into pUC119. This mini-library was screened by colony hybridization using two *Hin*d III fragments from pAQPR80 (Table 2.1); a 1.9 kb (containing most of *psaA*) and a 1.4 kb *Hin*d III fragment (containing the 3' half of *psaB*). Two hybridizing clones were isolated: one contained a 1.8 kb *Kpn* I fragment (pLS18) and the other contained a 0.9 kb *Kpn* I fragment (pLS19) (Figure 2.1). Plasmids pLS18 and pLS19 were partially sequenced and, by comparison to the *Synechococcus* 7002 sequence, were verified to encode portions of *psaA* and *psaB*. Inserts from pLS18 and pLS19 and a 0.5 kb *Eco*R I-*Hin*d III fragment from pAQPR80 (encoding the 5' end of *psaA*) (Table 2.1) were then used as probes in screening a λEMBL3 library of a partial *Sau* 3A digest of *Synechocystis* 6803 DNA to recover full-length clones. Several hybridizing λ clones were isolated and their DNA purified, mapped, and subcloned into pUC118 or pUC119. The maps of two of the λ clones, 311 and 121, and their subclones are shown in Figure 2.1. Southern analysis of digested genomic *Synechocystis* 6803 DNA, probed with the inserts from pLS18 and pLS19, revealed only one large or a few small hybridizing fragments, as with pAQPR80, indicating *psaA* and *psaB* are present in single copy in the *Synechocystis* 6803 genome (data not shown).

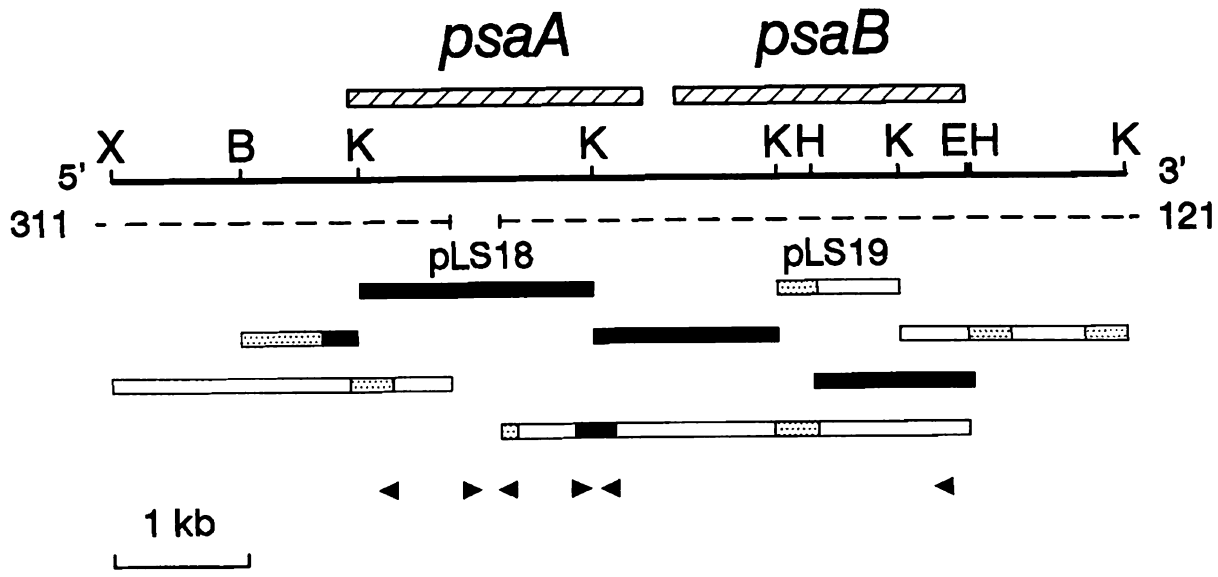

Figure 2.1. Restriction map and sequencing strategy of the *Synechocystis* 6803 *psaA* and *psaB* operon. Hatched boxes represent the protein coding regions of *psaA* and *psaB*. A partial restriction map is shown on the solid line: X, *Xba* I; B, *Bgl* II; K, *Kpn* I; H, *Hin*d III; E, *Eco*R I. Dashed lines represent the insert regions from two of the λ clones (311 and 121). Boxes below the dashed lines represent clones from the mini-library or sub-clones of the λ clones: black regions were sequenced from both strands, stippled regions were sequenced from one strand. Inserts from the plasmids pLS18 and pLS19 are labelled above the boxes. Triangles represent oligonucleotides used as sequencing primers and in PCR.

DNA sequence

The sequencing strategy is shown in Figure 2.1. Sequencing revealed a single base-pair deletion in one of the original clones from the mini-library (pLS18), evidenced by a disruption of the reading frame in a highly conserved region of *psaA*. Unfortunately, this region was not overlapped by any of the λ clones. Therefore, the PCR technique was utilized to amplify the region between two of the sequencing primers from genomic *Synechocystis* 6803 DNA. The PCR product was purified, subcloned into pUC118, and sequenced, verifying the true sequence and our hypothesis of a deletion in our cloned fragment in pLS18.

The DNA and deduced amino acid sequence of the *Synechocystis* sp. PCC 6803 *psaA* and *psaB* genes and flanking regions is shown in Figure 2.2. The genes are arranged in tandem with *psaA* upstream of *psaB*. The *psaA* gene consists of 2256 nucleotides, which would encode a protein 751 amino acids long with a predicted molecular mass of 82.9 kDa. The *psaB* gene is 2196 nucleotides long and would encode a protein with 731 amino acids and a predicted molecular mass of 81.3 kDa. The region between the genes is 245 nucleotides long, with no apparent open reading frame or inverted repeat sequences. The *Synechocystis* 6803 *psaA* gene shows 78.0% DNA sequence identity to the *Synechococcus* 7002 *psaA* gene (Cantrell and Bryant, 1987) and 69.4% DNA identity to the spinach *psaA* gene (Kirsch *et al.,* 1986). The *Synechocystis* 6803 *psaB* gene has 81.1% DNA sequence identity to the *Synechococcus* 7002 *psaB* gene (Cantrell and Bryant, 1987) and 69.4% DNA

Figure 2.2. Nucleotide and deduced amino acid sequences of the *Synechocystis* 6803 *psaA* and *psaB* operon. The DNA sequence is numbered on the left; the deduced amino acid sequence is numbered on the right. Sequences showing similarity to *E. coli* promoters and ribosome binding sites are underlined. The *psaA* gene extends from nucleotide 1 to 2256. The *psaB* gene extends from nucleotide 2502 to 4697. The amino acid sequence FPCDGPGRGGTC, conserved in PsaA and PsaB and including cysteines thought to bind F_X, as well as the conserved leucines which may form a leucine zipper, upstream of those cysteines, are enclosed in boxes. The DNA sequence data reported appear in the EMBL/GenBank Database under the accession number X58825.

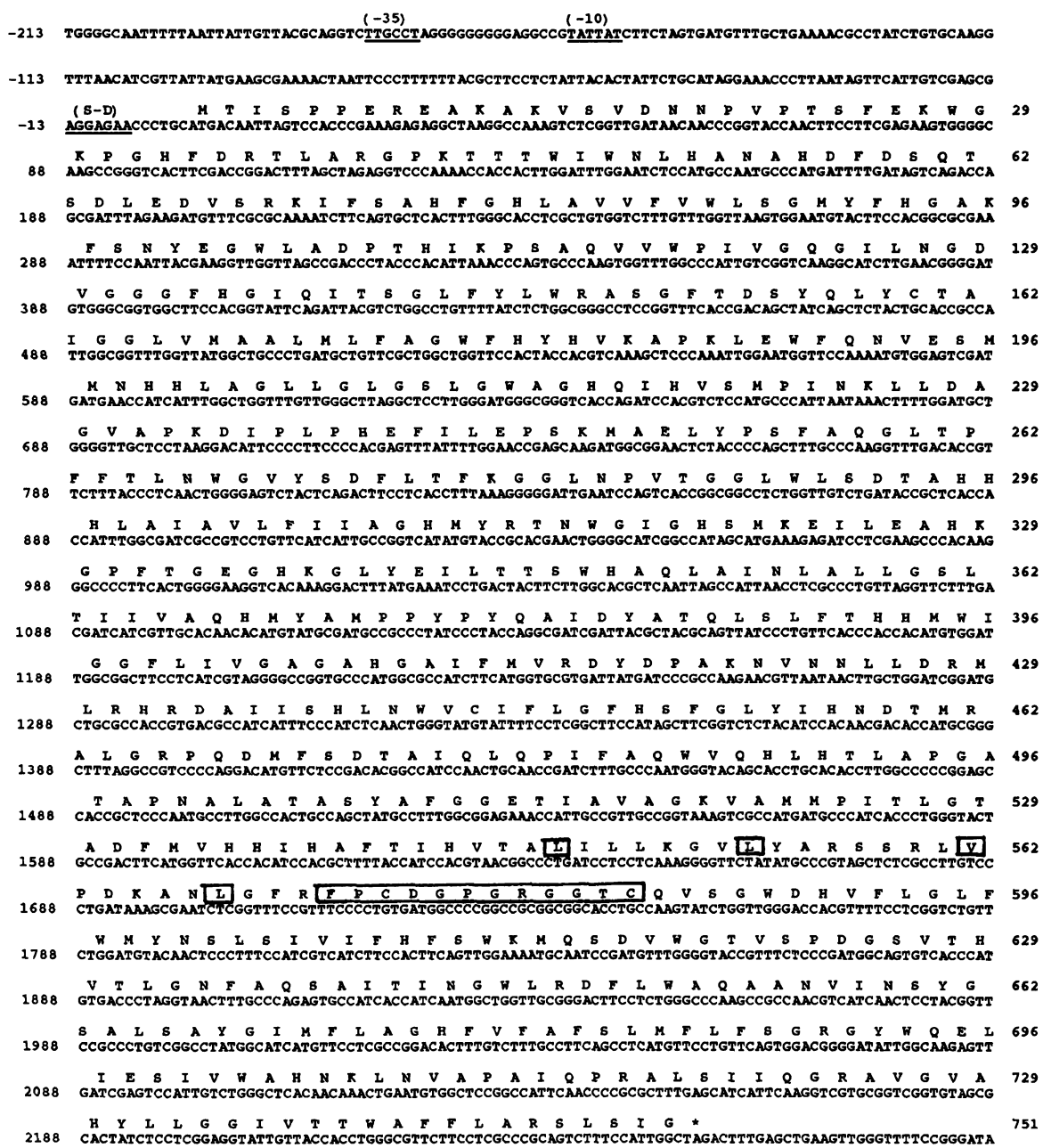
(-35)
(-10)
-213 TGGGGCAATTTTTAATTATTGTTACGCAGGTCTTGCCTAGGGGGGGGGAGGCCGTATTATCTTCTAGTGATGTTTGCTGAAAACGCCTATCTGTGCAAGG
-113 TTTAACATCGTTATTATGAAGCGAAAACTAATTCCCTTTTTTACGCTTCCTCTATTACACTATTCTGCATAGGAAACCCTTAATAGTTCATTGTCGAGCG
(S-D) M T I S P P E R E A K A K V S V D N N P V P T S F E K W G 29
-13 AGGAGAACCCTGCATGACAATTAGTCCACCCGAAAGAGAGGCTAAGGCCAAAGTCTCGGTTGATAACAACCCGGTACCAACTTCCTTCGAGAAGTGGGGC
K P G H F D R T L A R G P K T T T W I W N L H A N A H D F D S Q T 62
88 AAGCCGGGTCACTTCGACCGGACTTTAGCTAGAGGTCCCAAAACCACCACTTGGATTTGGAATCTCCATGCCAATGCCCATGATTTTGATAGTCAGACCA
S D L E D V S R K I F S A H F G H L A V V F V W L S G M Y F H G A K 96
188 GCGATTTAGAAGATGTTTCGCGCAAAATCTTCAGTGCTCACTTTGGGCACCTCGCTGTGGTCTTTGTTTGGTTAAGTGGAATGTACTTCCACGGCGCGAA
F S N Y E G W L A D P T H I K P S A Q V V W P I V G Q G I L N G D 129
288 ATTTTCCAATTACGAAGGTTGGTTAGCCGACCCTACCCACATTAAACCCAGTGCCCAAGTGGTTTGGCCCATTGTCGGTCAAGGCATCTTGAACGGGGAT
V G G G F H G I Q I T S G L F Y L W R A S G F T D S Y Q L Y C T A 162
388 GTGGGCGGTGGCTTCCACGGTATTCAGATTACGTCTGGCCTGTTTTATCTCTGGCGGGCCTCCGGTTTCACCGACAGCTATCAGCTCTACTGCACCGCCA
I G G L V M A A L M L F A G W F H Y H V K A P K L E W F Q N V E S M 196
488 TTGGCGGTTTGGTTATGGCTGCCCTGATGCTGTTCGCTGGCTGGTTCCACTACCACGTCAAAGCTCCCAAATTGGAATGGTTCCAAAATGTGGAGTCGAT
M N H H L A G L L G L G S L G W A G H Q I H V S M P I N K L L D A 229
588 GATGAACCATCATTTGGCTGGTTTGTTGGGCTTAGGCTCCTTGGGATGGGCGGGTCACCAGATCCACGTCTCCATGCCCATTAATAAACTTTTGGATGCT
G V A P K D I P L P H E F I L E P S K M A E L Y P S F A Q G L T P 262
688 GGGGTTGCTCCTAAGGACATTCCCCTTCCCCACGAGTTTATTTTGGAACCGAGCAAGATGGCGGAACTCTACCCCAGCTTTGCCCAAGGTTTGACACCGT
F F T L N W G V Y S D F L T F K G G L N P V T G G L W L S D T A H H 296
788 TCTTTACCCTCAACTGGGGAGTCTACTCAGACTTCCTCACCTTTAAAGGGGGATTGAATCCAGTCACCGGCGGCCTCTGGTTGTCTGATACCGCTCACCA
H L A I A V L F I I A G H M Y R T N W G I G H S M K E I L E A H K 329
888 CCATTTGGCGATCGCCGTCCTGTTCATCATTGCCGGTCATATGTACCGCACGAACTGGGGCATCGGCCATAGCATGAAAGAGATCCTCGAAGCCCACAAG
G P F T G E G H K G L Y E I L T T S W H A Q L A I N L A L L G S L 362
988 GGCCCCTTCACTGGGGAAGGTCACAAAGGACTTTATGAAATCCTGACTACTTCTTGGCACGCTCAATTAGCCATTAACCTCGCCCTGTTAGGTTCTTTGA
T I I V A Q H M Y A M P P Y P Y Q A I D Y A T Q L S L F T H H M W I 396
1088 CGATCATCGTTGCACAACACATGTATGCGATGCCGCCCTATCCCTACCAGGCGATCGATTACGCTACGCAGTTATCCCTGTTCACCCACCACATGTGGAT
G G F L I V G A G A H G A I F M V R D Y D P A K N V N N L L D R M 429
1188 TGGCGGCTTCCTCATCGTAGGGGCCGGTGCCCATGGCGCCATCTTCATGGTGCGTGATTATGATCCCGCCAAGAACGTTAATAACTTGCTGGATCGGATG
L R H R D A I I S H L N W V C I F L G F H S F G L Y I H N D T M R 462
1288 CTGCGCCACCGTGACGCCATCATTTCCCATCTCAACTGGGTATGTATTTTCCTCGGCTTCCATAGCTTCGGTCTCTACATCCACAACGACACCATGCGGG
A L G R P Q D M F S D T A I Q L Q P I F A Q W V Q H L H T L A P G A 496
1388 CTTTAGGCCGTCCCCAGGACATGTTCTCCGACACGGCCATCCAACTGCAACCGATCTTTGCCCAATGGGTACAGCACCTGCACACCTTGGCCCCCGGAGC
T A P N A L A T A S Y A F G G E T I A V A G K V A M M P I T L G T 529
1488 CACCGCTCCCAATGCCTTGGCCACTGCCAGCTATGCCTTTGGCGGAGAAACCATTGCCGTTGCCGGTAAAGTCGCCATGATGCCCATCACCCTGGGTACT
A D F M V H H I H A F T I H V T A L I L L K G V L Y A R S S R L V 562
1588 GCCGACTTCATGGTTCACCACATCCACGCTTTTACCATCCACGTAACGGCCCTGATCCTCCTCAAAGGGGTTCTATATGCCCGTAGCTCTCGCCTTGTCC
P D K A N L G F R F P C D G P G R G G T C Q V S G W D H V F L G L F 596
1688 CTGATAAAGCGAATCTCGGTTTCCGTTTCCCCTGTGATGGCCCCGGCCGCGGCGGCACCTGCCAAGTATCTGGTTGGGACCACGTTTTCCTCGGTCTGTT
W M Y N S L S I V I F H F S W K M Q S D V W G T V S P D G S V T H 629
1788 CTGGATGTACAACTCCCTTTCCATCGTCATCTTCCACTTCAGTTGGAAAATGCAATCCGATGTTTGGGGTACCGTTTCTCCCGATGGCAGTGTCACCCAT
V T L G N F A Q S A I T I N G W L R D F L W A Q A A N V I N S Y G 662
1888 GTGACCCTAGGTAACTTTGCCCAGAGTGCCATCACCATCAATGGCTGGTTGCGGGACTTCCTCTGGGCCCAAGCCGCCAACGTCATCAACTCCTACGGTT
S A L S A Y G I M F L A G H F V F A F S L M F L F S G R G Y W Q E L 696
1988 CCGCCCTGTCGGCCTATGGCATCATGTTCCTCGCCGGACACTTTGTCTTTGCCTTCAGCCTCATGTTCCTGTTCAGTGGACGGGGATATTGGCAAGAGTT
I E S I V W A H N K L N V A P A I Q P R A L S I I Q G R A V G V A 729
2088 GATCGAGTCCATTGTCTGGGCTCACAACAAACTGAATGTGGCTCCGGCCATTCAACCCCGCGCTTTGAGCATCATTCAAGGTCGTGCGGTCGGTGTAGCG
H Y L L G G I V T T W A F F L A R S L S I G * 751
2188 CACTATCTCCTCGGAGGTATTGTTACCACCTGGGCGTTCTTCCTCGCCCGCAGTCTTTCCATTGGCTAGACTTTGAGCTGAAGTTGGGTTTTCCGGGATA

Figure 2.2

Figure 2

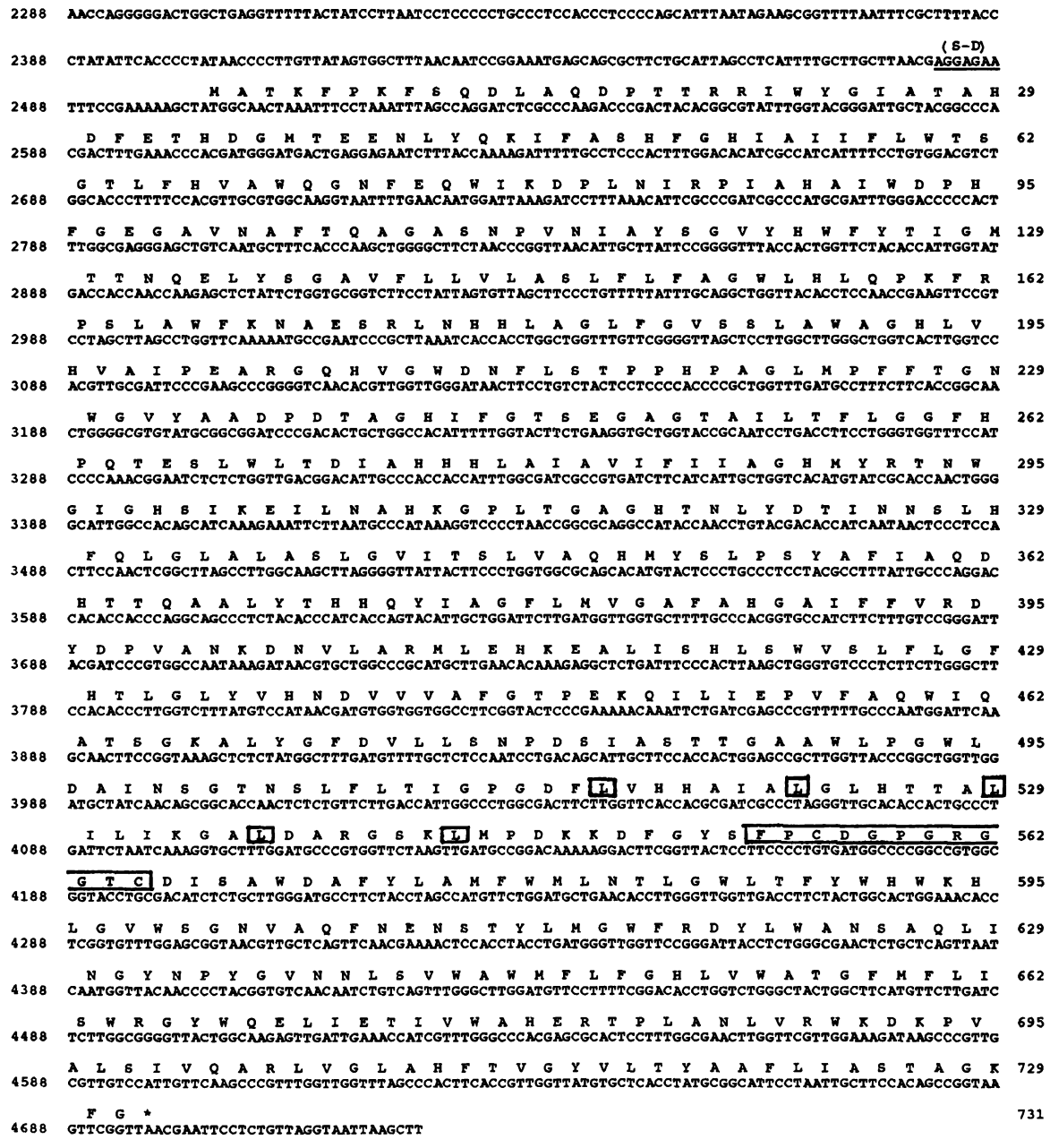

Figure 2.2 (cont.)

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identity to the spinach *psaB* gene (Kirsch *et al.,* 1986). Seven nucleotides upstream from the start of *psaA* and fifteen nucleotides upstream from the start of *psaB* is the sequence AGGAGAA, which has similarity to the Shine-Dalgarno sequence and may constitute a ribosome binding site (Shine and Dalgarno, 1974). A sequence (TATTAT) similar to the *E. coli* consensus -10 sequence (TATAAT) lies 154 nucleotides upstream of the start of *psaA* gene, while a sequence (TTGCCT) similar to the *E. coli* -35 sequence (TTGACA) lies 17 nucleotides upstream from the potential -10 sequence (Hawley and McClure, 1983). Partial nucleotide sequence obtained for the region 3' to the *psaB* gene includes G-C-rich inverted repeats that may form a stem-loop structure followed by a series of thymines. This type of structure is involved in transcription termination in *E. coli* (Rosenberg and Court, 1979). The partial sequence data from the region 3' to *psaB* show no homology to *rps14* from spinach (Kirsch *et al.,* 1986) or to ORF128 from *Synechococcus* 7002 (Cantrell and Bryant, 1987), which have been found downstream of *psaB* in those organisms.

psaA-psaB deduced amino acid sequence

Specific residues as well as predicted secondary structures are conserved in the *Synechocystis* 6803 *psaA-psaB* deduced protein sequences. The predicted amino acid sequence of the *psaA* gene (PsaA) is 88.4% identical to the *Synechococcus* 7002 PsaA (Cantrell and Bryant, 1987) and 80.9% identical to the spinach PsaA (Kirsch *et al.,* 1986). The *Synechocystis* 6803 *psaB*

deduced a
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deduced amino acid sequence (PsaB) is 92.8% identical to the *Synechococcus* 7002 PsaB (Cantrell and Bryant, 1987) and 79.6 % identical to the spinach PsaB (Kirsch *et al.,* 1986). Eleven membrane-spanning α-helices are predicted for both PsaA and PsaB, as has been predicted for previously characterized sequences (Cantrell and Bryant, 1987; Fish *et al.,* 1985; Kirsch *et al.,* 1986; Lehmbeck *et al.,* 1986). Also conserved in both deduced protein sequences is the sequence FPCDGPGRGGTC (residues 572 to 583 in PsaA, 554 to 565 in PsaB), which is absolutely conserved in the deduced amino acid sequences of all of the *psaA* and *psaB* genes sequenced to date. On the immediate amino-terminal side of this sequence in PsaA and PsaB are conserved series of leucine residues (four leucines in PsaA, five in PsaB), each leucine spaced seven amino acids apart. These regions are predicted to form α-helices, each with a string of leucines, that would have the potential to interact with each other, forming a leucine zipper (Kössel *et al.,* 1990; Webber and Malkin, 1990).

mRNA accumulation in cells grown with glucose

Northern blots of total RNA from *Synechocystis* 6803 were hybridized with probes encoding *psaA*, *psaB*, *psbA2*, 16S rRNA, *psaD*, *psbD2*, and *rbcL*, as well as a probe encoding 1.0 kb 3' to *psaB* (Table 2.1). RNA was isolated from cells grown in continuous light; cells grown in the light with glucose, then put in total darkness for 24, 30, or 48 h; and from cells grown under LAHG conditions (Anderson and McIntosh, 1991). Autoradiographs from hybridizations to a representative northern blot with samples from each of the

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above conditions are shown in Figure 2.3. Both the *psaA* and *psaB* probes hybridized to a 5.0-kb transcript, indicating they are co-transcribed. The *psaA* probe also hybridized to a 2.4-kb message, while the *psaB* probe also hybridized to a 2.1-kb message. Both probes hybridized to a smear of bands smaller than 5.0 kb. The fragment encoding *psbA2* hybridized to a 1.2-kb message that may be a transcript from one or both of *psbA2* or *psbA3*. A fragment of the *rrn* operon from *Synechococcus* 7942 specific for 16S rRNA hybridized to a 1.5-kb transcript. The *psaD* probe from *Synechocystis* 6803, encoding subunit II of PSI, hybridized to a single message of 0.5 kb. The *psbD2* probe hybridized strongly to the 1.2-kb transcript from *psbD2*, but also hybridized weakly to the 2.5-kb transcript from the overlapping *psbD1-psbC* genes. The probe for *rbcL* from *Synechocystis* 6803 hybridized to a 2.6-kb transcript, a 2.0-kb message, and a smear of transcripts smaller than 2.6 kb. The 2.6-kb transcript represents the full-length message from the *rbcLS* operon, which encodes the large and small subunits of Rubisco.

In order to quantify the steady-state levels of mRNA accumulation for *psaA-psaB* and *psbA*, radioactivity in the region of the bands was measured directly from the blots using a Betascope machine. These values were then normalized based on the quantification of hybridization of the 16S rRNA probe. Quantification of hybridization of the *psaA* probe was measured in the regions of the 5.0-kb full-length *psaA-psaB* message and in the region of the 2.4-kb *psaA*-specific message. Values for at least one quantification from at

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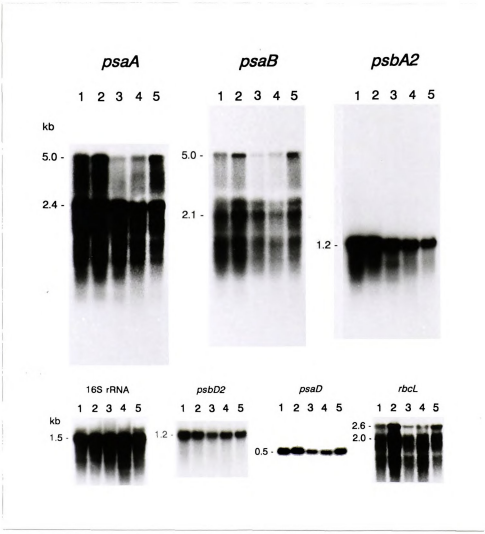

Figure 2.3. Autoradiographs of a single northern blot, stripped and reprobed. Probes used are indicated above each panel and are described in Table 2.1. The upper three panels are aligned in the same scale. The lower four panels are not in alignment. Sizes of bands are in kb and were determined by comparison to an ethidium bromide-stained RNA ladder. Lanes are: 1, light-grown control; 2, 24 h dark with glucose; 3, 30 h dark with glucose; 4, 48 h dark with glucose; 5, LAHG conditions. Exposure times were different for each autoradiograph.

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least two preparations for each condition were averaged and are expressed as a percentage of the accumulation in the light-grown samples (Figure 2.4). Accumulation of the 5.0-kb *psaA-psaB* transcript drops to 87% of the level in the light after 24 h in the dark, 47% after 30 h, then back up to 82% after 48 h, while the steady-state level in LAHG cells is 80% of light-grown. However, the accumulation of the 2.4-kb *psaA* message increases to 152% of the level in the light after 24 h, but drops to 97% after 30 h, 107% after 48 h, while the steady-state level in LAHG cells is 85% of the level in light-grown cells. The accumulation of the 1.2-kb *psbA* message steadily decreases through the dark incubation: 68% after 24 h, 50% after 30 h, and 41% after 48 h, while the steady-state level of this *psbA* message in LAHG cells is 28% of that in light-grown cells. Although the hybridization of the other probes used was not quantified, none showed a significant and repeatable change upon growth in the dark with glucose.

mRNA accumulation in cells grown without glucose

RNA was isolated from duplicate cultures of *Synechocystis* 6803 grown to early exponential phase in the light without glucose, then put in complete darkness for 48 h. The final OD_{730} of the culture was lower than when the dark incubation started, and the yield of RNA from these preparations was significantly lower than for cells grown with glucose. When the RNA in the formaldehyde gel was stained with ethidium bromide and visualized under UV light, the samples from cells grown without glucose were indistinguishable from

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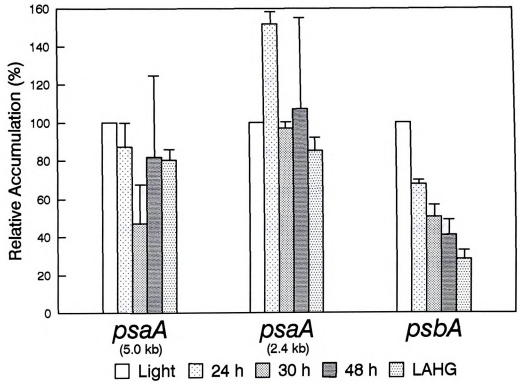

Figure 2.4. Relative levels of mRNA accumulation in *Synechocystis* 6803 cells. Values are averages of at least one quantification of hybridization to at least two preparations from each condition and are expressed as a percentage of the light-grown control. Values were normalized based on the hybridization of the16S rRNA probe. The left group is a quantification of the 5.0-kb *psaA-psaB* transcript. The middle group is a quantification of the 2.4-kb *psaA*-specific transcript. The right group is a quantification of the 1.2-kb *psbA* transcript. Bars are: Light, light-grown control; 24 h, 24 h dark with glucose; 30 h, 30 h dark with glucose; 48 h, 48 h dark with glucose; LAHG, light-activated heterotrophic growth conditions.

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those from cells grown with glucose (data not shown). Autoradiographs of a representative lane from a northern blot with this RNA hybridized to the *psaA*, *psbA2*, *psbD2*, *rbcL*, and 16S rRNA probes are shown in Figure 2.5. By inspection of autoradiographs, the level of hybridization to the mRNA from cells lacking glucose was significantly lower than the level of hybridization to mRNA from either the 48 h dark with glucose sample or the LAHG sample, while the hybridization of the 16S rRNA probe appeared to be approximately equal. The *psaA* probe hybridized to a smear of transcripts of 3.0 kb and smaller, with bands of 2.4, 1.6, 1.1, and 0.7 kb. The *psbA2* probe hybridized to the expected 1.2-kb message and a 0.6-kb transcript. The *psbD2* probe hybridized strongly to the 1.2-kb *psbD2* transcript and very weakly to the 2.5-kb *psbD1-psbC* message. Hybridization of the *rbcL* probe to a smear of transcripts 2.5 kb and smaller was nearly undetectable. The 16S rRNA probe hybridized to the expected 1.5-kb transcript.

DISCUSSION

The use of *Synechocystis* sp. PCC 6803 as a model system for the study of oxygenic photosynthesis has been well documented (Debus *et al.,* 1988; Jansson *et al.,* 1987; Williams, 1988). In order to broaden the analysis of the biogenesis and structure/function relationships of PSI in *Synechocystis* 6803, we have cloned the *psaA* and *psaB* genes for use as probes to study gene expression and for targeted mutagenesis. As a part of a study to determine whether the photosystems assemble in the dark, a quantitative analysis of the

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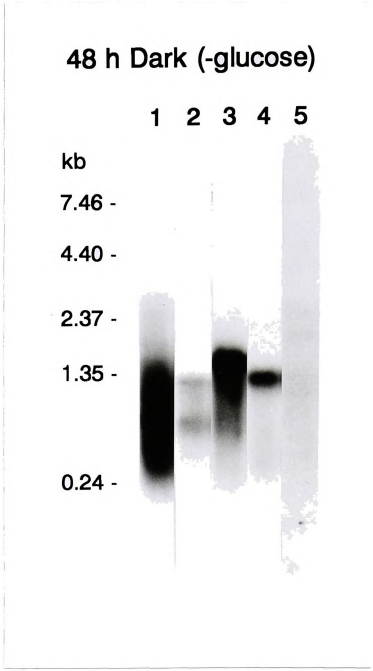

Figure 2.5. Autoradiographs of a single lane from a northern blot (48 h dark without glucose), stripped and reprobed. Size labels (in kb) are from an ethidium bromide-stained RNA ladder. Probes used: 1, *psaA*; 2, *psbA2*; 3, 16S rRNA; 4, *psbD2*; 5, *rbcL*. Probes used are described in Table 2.1. Exposure times were different for each autoradiograph.

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expression of photosynthesis genes in the dark was performed. Although the *psaA* and *psaB* genes have been cloned and sequenced from a cyanobacterium as well as multiple plant and algal species, the recent discovery of heterotrophic growth conditions for *Synechocystis* 6803 (Anderson and McIntosh, 1991) makes mutagenesis of the PSI core tractable in this organism.

The *psaA* and *psaB* genes from *Synechocystis* 6803 are highly homologous to the genes from *Synechococcus* 7002 and only slightly less homologous to the genes cloned from higher-plant chloroplasts. The *psaA* gene is homologous to the *psaB* gene, with long stretches of absolute amino acid conservation and 56.9% DNA sequence identity in *Synechocystis* 6803. This suggests one of these genes arose from a duplication of the other prior to the origin of chloroplasts. Fundamental differences in the *psaA-psaB* operon structure from cyanobacteria and higher plant chloroplasts do exist. In the plant sequences, there are only 25 bp between the *psaA* and *psaB* genes, while in *Synechococcus* 7002 this region is 173 bp, and in *Synechocystis* 6803 it is 245 bp. There is no apparent open reading frame in the intergenic region of *Synechocystis* 6803, while there is a short open reading frame in *Synechococcus* 7002. Also, in tobacco, rice, and spinach, the gene for ribosomal protein S14 (*rps14*) is found downstream of *psaB* and is co-transcribed with *psaA* and *psaB*. A fragment encoding 1.0 kb immediately downstream of *psaB* was used as a probe of northern blots, but no hybridization was detected (data not shown). Neither *Synechocystis* 6803 nor *Synechococcus* 7002 have *rps14* immediately downstream of *psaB*, and in *Synechocystis* 6803, this region may not be

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The predicted amino acid sequences of *psaA* and *psaB* from *Synechocystis* 6803 are highly homologous to those from *Synechococcus* 7002 and higher plants. Eleven membrane-spanning α-helices are predicted for both PsaA and PsaB. An interesting feature of the eighth helix in both PsaA and PsaB is the presence of conserved leucine residues, spaced seven amino acids apart. It has been proposed that these helices may interact to form a leucine zipper, pulling PsaA and PsaB together (Kössel *et al.,* 1990; Webber and Malkin, 1990). These helices are immediately adjacent to highly conserved regions containing the four cysteines thought to bind the 4Fe-4S center F_X (Golbeck and Bryant, 1991). We plan to use site-directed mutagenesis of the leucines described above to address the leucine zipper hypothesis and assess its role in the assembly of F_X and of the PSI core. In addition, we plan to mutate one or more of the conserved cysteines to confirm their role as ligands to F_X, to assess the importance of F_X in maintaining the integrity of the core heterodimer, and to answer questions about the role of F_X in electron transport.

Hybridization patterns of *psaA* and *psaB* to northern blots indicate these genes are co-transcribed, as both genes hybridize to a 5.0 kb transcript. However both genes also hybridize to smaller transcripts that may represent the products of processing in the intergenic region, yielding transcripts that only encode *psaA* or *psaB*. The lack of an open reading frame in the relatively long intergenic region may leave the transcript susceptible to processing, as this

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region apparently would not be bound to ribosomes. Probes for *psaA* and *psaB* hybridize to a smear of transcripts smaller than 5.0 kb. Smearing was not seen in hybridizations with other probes, suggesting this is not the result of general degradation of the RNA sample, but perhaps due to rapid turnover of this message. A similar complex pattern of hybridization has been seen in maize (Rodermel and Bogorad, 1985) and in tobacco (Meng *et al.,* 1988). The half-life of the *psaA-psaB* transcript has not been measured in cyanobacteria or chloroplasts.

The accumulation of transcripts from genes encoding core proteins of PSI and PSII was quantified for three time points of incubation in total darkness with glucose, for LAHG conditions, and for light-grown cells. These quantifications were averaged from multiple trials from at least two preparations of each type. The doubling time for cells in continuous light is about 12 h (Williams, 1988), while the doubling time for cells in LAHG conditions is 36 h (Anderson and McIntosh, 1991). When cells were transferred from the light to total darkness, their growth rate slowed to approach that for LAHG conditions, presumably due to a change in energy status. After approximately 48 h without a light pulse, the cells would stop dividing. The accumulation of the *psaA-psaB* transcript drops only slightly through the dark incubation, with a steady-state level of 80% of that of light-grown cells in the LAHG sample. The accumulation of the *psaA*-specific transcript generally shows the same pattern as the full-length transcript. The accumulation of *psbA* transcript shows a consistent decline through the dark

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incubation to a level in LAHG cells 28% of that in light-grown cells. These changes in message accumulation may not be due to transcriptional regulation, but rather to changes in mRNA stability. Also, any changes in message accumulation seen under these conditions cannot be attributed solely to light regulation, but may be the result of the difference in energy status when cells are grown in the dark. It is clear that transcripts of the photosynthesis genes assayed in this experiment do accumulate in cells grown in the dark.

Mohamed and Jansson (Mohamed and Jansson, 1989) published results of an experiment using *Synechocystis* 6803 in which they saw no accumulation of *psbA*, *psbD*, or *rbcL* mRNA in cells put in the dark for 48 h without glucose. Our results from a duplication of this experiment indicate that transcripts from these genes and *psaA-psaB* do accumulate in cells grown in the dark without glucose, but the mRNA is degraded (Figure 2.5). The OD_{730} of the cultures actually decreased after the 48 h incubation, and the yield of RNA was much lower than from cultures grown in complete darkness with glucose. Anderson and McIntosh (1991) showed that *Synechocystis* 6803 does not divide in the dark without supplemental glucose. We would conclude that the mRNA of cells grown under these conditions has been degraded and is not satisfactory for the study of gene expression.

The presence of transcripts from photosynthesis genes in cells grown in total darkness indicates that either transcription continues in the dark or that the transcripts are stable for as long as 48 h. In either case, the mRNA is available for the synthesis and assembly of photosynthesis proteins. The

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pattern of hybridization to *psaA-psaB* suggests this message is rapidly turned over, thus transcription of this operon probably continues in the dark at near light-grown rates. The half-life of *psbA* in *Synechococcus* 6301 has been estimated to be approximately 120 min (Lönneborg *et al.,* 1988). If this is similar in *Synechocystis* 6803, the rate of transcription of *psbA* may drop shortly after the shift to the dark, but the *psbA* transcript pool decreases gradually. Changes in the *psbA* transcript pool have been observed when *Synechocystis* 6803 (Mohamed and Jansson, 1989) or *Synechococcus* 6301 (Lönneborg *et al.,* 1988) are grown in high light. Transcriptional regulation of *psbA* may be one of the ways this organism alters its photosynthetic apparatus to adjust to available light conditions.

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CHAPTER 3

Targeted genetic inactivation of the photosystem I reaction center in the cyanobacterium *Synechocystis* sp. PCC 6803

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INTRODUCTION

Photosystem (PS) I is a multi-subunit, membrane-bound protein complex that functions as a light-driven, plastocyanin:ferredoxin oxidoreductase. PSI operates in both cyclic electron flow, driving ATP synthesis, and in linear electron flow, which drives ATP synthesis and the generation of reducing power as NADPH. Light energy is captured by the PSI antenna and is funneled to a chlorophyll (chl) *a* special-pair, P_{700}, which passes an energized electron to A_0 (a specialized chl *a*). P_{700}^+ is then reduced by plastocyanin, which may be replaced by cytochrome c_{553} in cyanobacteria (Sandmann, 1986). Electron transfer proceeds from A_0 to A_1 (most likely a phylloquinone), and then to F_X (a [4Fe-4S] center), which donates electrons to F_A and/or F_B (both [4Fe-4S]

centers), the terminal acceptors of PSI. F_A and/or F_B reduce the soluble electron carrier ferredoxin, which reduces $NADP^+$ to NADPH, catalyzed by the ferredoxin:$NADP^+$ oxidoreductase (for a recent review of PSI, see (Golbeck and Bryant, 1991)). The electron transfer components P_{700}, A_0, A_1, and F_X are bound to a heterodimer of homologous 82-83 kDa polypeptides, PsaA and PsaB (Golbeck *et al.,* 1988; Golbeck and Bryant, 1991), encoded by the *psaA* and *psaB* genes, which have been mapped to the plastome of plants and algae (Westhoff *et al.,* 1983; Girard-Bascou, 1987b). Centers F_A and F_B are bound to a 9 kDa polypeptide, PsaC, also encoded in the plastome by *psaC* (Høj *et al.,* 1987). Functions have been proposed for two of the eight additional known PSI proteins: PsaD is thought to be involved in ferredoxin binding (Zilber and Malkin, 1988), while PsaF may be involved in plastocyanin binding (Wynn and Malkin, 1988).

Mutants with aberrant PSI have been isolated in *Chlamydomonas reinhardtii* (Chua *et al.,* 1975; Girard *et al.,* 1980; Girard-Bascou, 1987b), maize (Cook and Miles, 1990; Miles *et al.,* 1979), barley (Møller *et al.,* 1980), *Antirrhinum majus* (Herrmann, 1971), *Scenedesmus* (Gregory *et al.,* 1971), and *Oenothera* (Fork and Heber, 1968). Two nuclear mutants of *C. reinhardtii* described by (Chua *et al.,* 1975) lack P_{700} activity and the P_{700}- apoproteins, yet still maintain wild-type levels of PSII activity and PSII core proteins. Further analysis of those mutants and 23 other nuclear mutants of *C. reinhardtii* deficient in the PSI reaction center proteins revealed the loss of a group of small polypeptides that are enriched in PSI preparations from wild-type cells

(Girard *et al.,* 1980). Some of those nuclear mutations have been shown to block the *trans*-splicing of the *psaA* message in the chloroplast, thus abolishing PSI assembly (Choquet *et al.,* 1988; Goldschmidt-Clermont *et al.,* 1990). In addition, mutations that mapped to the *psaB* gene in the plastome of *C. reinhardtii* resulted in the loss of the PSI reaction center, as well as the same group of small polypeptides deficient in the nuclear mutants (Girard-Bascou *et al.,* 1987a). A nuclear mutant of barley, deficient in the PSI reaction center, also lacks at least two small proteins that are enriched in wild-type PSI particles, but maintains wild-type levels of PSII activity (Møller *et al.,* 1980). Similar plastome mutants of *Oenothera* (Fork and Heber, 1968) and *Antirrhinum* (Herrmann, 1971) and nuclear mutants of maize (Miles *et al.,* 1979) that are deficient in the PSI reaction center also retain PSII activity.

Targeted mutagenesis of PSI genes has not been accomplished in plants or algae; however, inactivation of genes for two peripheral PSI proteins has been achieved in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Chitnis *et al.,* 1989b; Chitnis *et al.,* 1989a). Inactivation of the *psaD* gene resulted in reduced growth rate under photoautotrophic conditions, but wild-type growth rate under photoheterotrophic conditions (with DCMU [N_1-(dichlorophenyl)-N_3-dimethyl urea] and glucose in the light) (Chitnis *et al.,* 1989a). When the *psaE* gene, encoding an 8-kDa protein with unknown function, was inactivated, only minor changes in PSI activity and photoautotrophic growth were observed (Chitnis *et al.,* 1989b). Similar results were obtained when *psaE* was inactivated in the cyanobacterium *Synechococcus*

sp. PCC 7002 (Bryant *et al.,* 1990).

Attempts at targeted mutagenesis of the *psaA-psaB* genes in *Synechococcus* sp. PCC 7002 have resulted in only partial segregation of the mutation (Zhao *et al.,* 1990), perhaps because selection was performed in the light, which gave a selective advantage to cells maintaining wild-type PSI. This problem is avoided by performing selection and segregation under heterotrophic growth conditions, which have recently been defined for *Synechocystis* 6803 (Anderson and McIntosh, 1991). Termed light-activated heterotrophic growth (LAHG), cells are grown with 5 mM glucose in complete darkness except for 5 min of light (40 μmol·m^{-2}·s^{-1}) every 24 h (Anderson and McIntosh, 1991). Here we describe the first complete segregation of a directed inactivation of *psaA* in *Synechocystis* 6803 by selection under LAHG conditions.

RESULTS

Construct interrupting *psaA*

A 1.8 kilobase pair (kb) *Kpn* I fragment, encoding most of the *psaA* gene from *Synechocystis* 6803 (Smart and McIntosh, 1991, see Chapter 2), in pUC119 (Vieira and Messing, 1987) (plasmid pLS18), was digested with *Nco* I and the resulting single-stranded DNA overhangs were digested briefly with mung bean nuclease. A 1.25-kb *Hin*c II fragment, encoding an aminoglycoside 3'-phosphotransferase from *Tn*903 conferring resistance to kanamycin (Km) (Oka *et al.,* 1981), was purified from pUC4K (Vieira and Messing, 1982; Taylor

and Rose, 1988) and ligated into the blunted *Nco* I site of pLS18. This was transformed into *Escherichia coli* DH5α (Gibco-BRL) and Km-resistant (Km^R) colonies were recovered. The resulting plasmid, pLS18K, had lost the *Nco* I site and the *Hinc* II sites flanking the Km^R cassette. A restriction map of the construct from pLS18K is shown in Figure 3.1.

Transformation of *Synechocystis* 6803 and selection conditions

Synechocystis 6803 that had been maintained under LAHG conditions for two subcultures was transformed with the plasmid pLS18K. Selection for Km^R colonies was performed under both mixotrophic (continuous light and 5 mM glucose) and LAHG conditions. Single Km^R colonies were streaked to at least five serial plates to obtain full segregation of the mutation. One Km^R colony recovered under LAHG conditions (ADK9) and one recovered under mixotrophic conditions (ALT6) were analyzed further.

Genetic mapping and segregation analysis

Southern blots of genomic digests from ADK9 were probed with the 1.8 kb *Kpn* I insert from pLS18 (Figure 3.2A). A restriction map of the *psaA-psaB* region is shown in Figure 3.1. In all the digests of ADK9 DNA, no wild-type copies of the *psaA* gene were detected (Figure 3.2A), even after prolonged exposure (not shown). Hybridization of *psaA* to a *Hind* III digest of ADK9

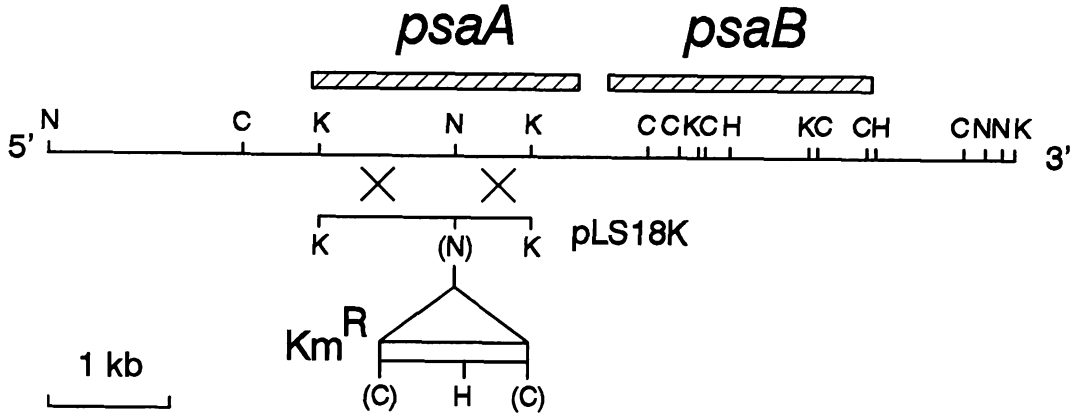

Figure 3.1. Restriction map of the insert region from plasmid pLS18K and of the *psaA-psaB* operon in *Synechocystis* 6803. Protein coding regions for *psaA* and *psaB* are shown as hatched boxes. Cloned *Synechocystis* 6803 DNA in pLS18K is indicated as a line, while the Km^R gene from pUC4K is indicated as a box. Potential regions of cross-over integration into the *Synechocystis* 6803 chromosome are shown as crosses. Restriction sites are: N, *Nco* I; C, *Hinc* II; K, *Kpn* I; H, *Hind* III. Sites in parentheses were destroyed in the construction of pLS18K.

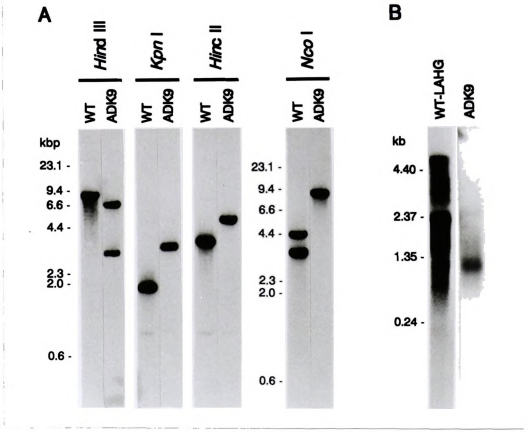

Figure 3.2. Autoradiographs of Southern and northern blots of wild type and ADK9. **A.** Autoradiographs of Southern blots of wild-type and ADK9 DNA probed with the insert from pLS18. Enzymes used for digestion are indicated. 7.5 μg of DNA were loaded per lane. The *Hind* III, *Kpn* I, and *Hinc* II digests were run on the same gel; the *Nco* I digest was run on a separate gel. Size markers were from λ digested with *Hind* III and are indicated in kilobase pairs (kbp). **B.** Autoradiographs of northern blots of WT-LAHG RNA (15 μg) and ADK9 RNA (15 μg) probed with the insert from pLS18. Size markers are from an RNA ladder (Gibco BRL) stained with ethidium bromide and are indicated in kb.

DNA revealed a new *Hin*d III site (within the Km^R cassette), creating fragments of 6.1 and 2.9 kb, rather than the wild-type 7.7 kb fragment. The *psaA* probe hybridized to an 8.7 kb *Nco* I fragment of ADK9 DNA, rather than to the 4.3 and 3.2 kb wild-type fragments, confirming the loss of this site by the insertion of the Km^R-cassette. Hybridization to *Hin*c II and *Kpn* I digests of ADK9 DNA showed an increase in the length of the hybridizing fragments due to the insertion of the Km^R gene. The wild-type *psaA* and *psaB* genes are arranged in tandem and are co-transcribed (Smart and McIntosh, 1991, see Chapter 2). Insertion of the Km^R cassette caused premature termination of transcription, yielding a 1.2 kb message, rather than the wild-type 5.0 kb message (Figure 3.2B).

DNA was isolated from ALT6 at three stages of segregation: after five serial platings under mixotrophic conditions (5 x MIXO), after five platings under mixotrophic and one plating under LAHG conditions (1 x LAHG), and after five platings under mixotrophic and five platings under LAHG conditions (5 x LAHG). Km selection was maintained for all subcultures. These DNA samples were digested with *Hin*d III, separated by electrophoresis, blotted, and hybridized with the *psaA* probe described above to determine the degree of segregation of the *psaA* mutation at each stage (Figure 3.3). In addition, the hybridization to fragments of 7.7, 6.1, and 2.9 kb was quantified using a Betascope to determine the percentage of hybridization to wild-type or mutant fragments (data not shown). The DNA from ALT6(5 x MIXO) has a high percentage of wild-type copies of *psaA* (Figure 3.3); quantification showed the

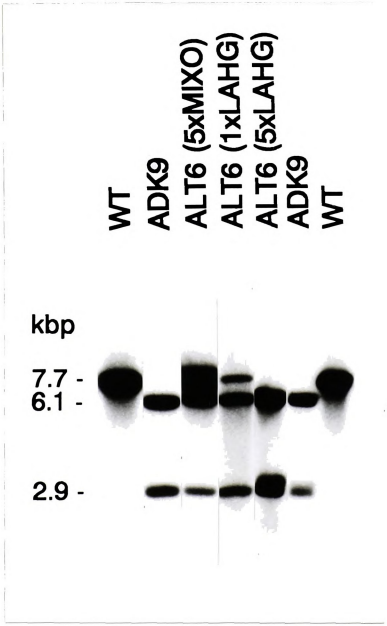

Figure 3.3. Autoradiographs of Southern blots of wild-type, ADK9, and ALT6 DNA. DNA was digested with *Hin*d III and probed with the insert from pLS18. 7.5 μg of DNA were loaded per lane. The four lanes on the left were run on the same gel; the three lanes on the right were run on a separate gel. DNA was isolated from ALT6 after 5 serial platings in mixotrophic conditions (5xMIXO), after 5 platings in mixotrophic and 1 plating in LAHG conditions (1xLAHG), and after 5 platings in mixotrophic and 5 platings in LAHG (5xLAHG). Sizes were estimated from λ digested with *Hin*d III and are indicated in kilobase pairs (kbp).

ratio of wild-type to mutant copies to be 3:1. After only one plate grown under LAHG conditions, the ratio of wild-type to mutant copies shifts dramatically to 1:3. Five serial platings under LAHG conditions is sufficient for segregation of the *psaA* mutation (Figure 3.3, 5 x LAHG).

Chlorophyll determination and oxygen evolution

The amount of chlorophyll in whole cells harvested at late log phase and in isolated membranes is presented in Table 3.1 for WT-MIXO, WT-LAHG, and ADK9 cells. The amount of chlorophyll per wild-type cell drops slightly more than four-fold when grown under LAHG conditions. The ADK9 mutant has less than half the chlorophyll per cell, when compared with WT-LAHG. The relative amounts of chlorophyll per mg protein in membranes from the three cell types approximately fits the same ratios as chlorophyll per cell (Table 3.1). The oxygen evolution rates of all three cell types, assayed in the presence of DCBQ, which accepts electrons from PSII in a DCMU-sensitive reaction (Rutherford, 1988), are approximately equal, when normalized to equal cell number (Table 3.1). The whole chain photosynthesis rates, measured with no added acceptor, are only slightly lower for WT-LAHG compared with WT-MIXO, on a per cell basis (Table 3.1). However, the oxygen evolution rate with no artificial acceptor for ADK9 is ≈4-fold lower than for WT-LAHG, when normalized to equal cell number (Table 3.1). Absorbance at 730 nm (OD_{730}) is a measure of the light scattering of a cell suspension and does not assay absorption of any particular pigment. The number of cells per ml,

Table 3.1. Chlorophyll determination and photosynthesis assays.

	WT-MIXO	WT-LAHG	ADK9
Chlorophyll determination			
Whole Cells[a]			
μg chl/(ml·O.D.$_{730}$)	3.66±0.63	0.81±0.31	0.37±0.09
Membranes[b]			
μg chl/(mg protein)	43.1±7.2	9.9±0.4	5.2±1.4
Oxygen evolution			
1 mM DCBQ[c]			
μmol O_2/(mg chl·h)	206±16	832±194	1690±306
μmol O_2/(l·O.D.$_{730}$·h)	753	674	625
No added acceptor[d]			
μmol O_2/(mg chl·h)	148±30	537±79	287±79
μmol O_2/(l·O.D.$_{730}$·h)	541	435	106

[a]Averages of at least five samples of each type.

[b]Averages of two samples of each type.

[c]Averages of at least 14 trials for each type.

[d]Averages of at least six trials for each type.

relative to OD_{730}, was approximately equal for all three cell type (data not shown), indicating that OD_{730} is a good estimate of cell number.

Growth characteristics

The growth of ADK9 was indistinguishable from that of wild-type cells under LAHG conditions (data not shown). However, after several attempts, ADK9 would not divide under continuous illumination of 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$, our standard conditions for growth of wild-type *Synechocystis* 6803 (Jansson *et al.*, 1987).

EPR spectra

Spectra from membranes isolated from WT-MIXO, WT-LAHG and ADK9 cells are shown in Figure 3.4. The spectra from illuminated wild-type membranes show a prominent feature with a linewidth of 0.8-1.0 milliTesla (mT) centered at g=2.0026, characteristic of signal I from P_{700}^+ (panel A) (Norris *et al.*, 1971). However, the signal from illuminated ADK9 membranes had a 2.0 mT linewidth centered at g=2.0040, typical of signal II from Y_D^+ (panels A and E) (Barry and Babcock, 1987). Signal II was detected from membranes of all three cell types after dark adaptation (panel B). In ADK9, there is no detectable signal I (panels A and E).

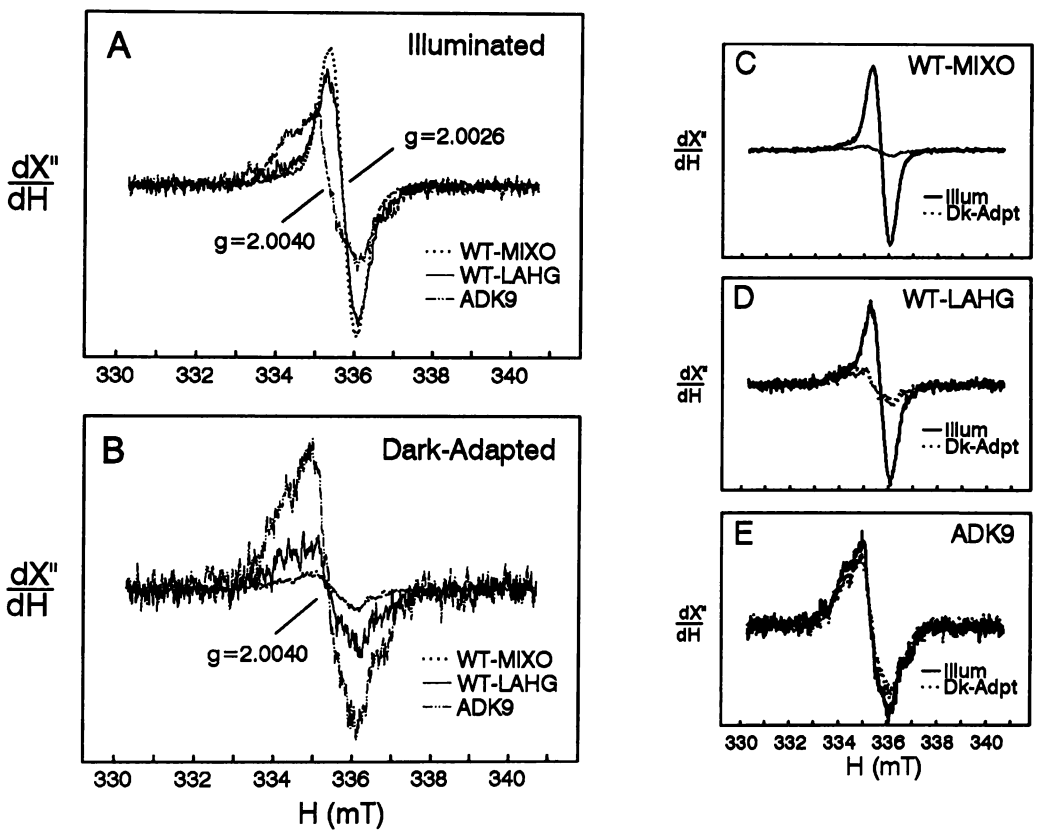

Figure 3.4. EPR spectra of thylakoid membranes from WT-MIXO, WT-LAHG, and ADK9. Illumination was provided by a high-intensity microscope lamp; dark-adaptation was for at least 10 min. Panel A contains spectra from all three cell types under illumination, panel B shows spectra from all three cell types after dark-adaptation. Panels C, D, and E contain the same spectra as in A and B presented as illuminated and dark-adapted for each cell type. Spectra are standardized to equal gain and to 1 mg chl·ml^{-1}.

Detection of proteins by antibodies

Thylakoid proteins from WT-MIXO, WT-LAHG, and ADK9 were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies raised to PSI proteins and to the D1 protein of PSII (Figure 3.5). Approximately equal protein was loaded in each lane, 120 μg protein representing 5 μg chl for WT-MIXO, 150 μg protein for WT-LAHG (1.5 μg chl), and 145 μg protein for ADK9 (0.75 μg chl). Antibodies raised to PsaA and PsaB from spinach cross-reacted to a diffuse band with apparent molecular mass of 60 - 65 kDa in both wild-type samples, but not in ADK9 (panel A). This anomalous behavior of the P_{700}-apoproteins in SDS-PAGE, migration at a lower molecular weight than the predicted 83 kDa, has been observed previously and is probably due to the highly hydrophobic nature of these proteins (Fish *et al.,* 1985). Antibodies raised to PsaD cross-reacted to protein of $\approx$15 kDa in both wild-type samples, both not in ADK9 (panel B). Similarly, antibodies raised to PsaC cross-reacted to a protein of $\approx$9 kDa in the wild-type samples (panel C), including a purified PSI preparation, but not in the ADK9 sample. The PsaC antibody cross-reacted to high molecular weight background bands in wild-type and ADK9 thylakoids, but not in a PSI preparation. With all three antibodies, development was allowed to continue until background bands appeared in the ADK9 lane, to ensure there was no signal from a specific cross-reaction in that lane. The intensity of signal from WT-MIXO was greater than from WT-LAHG for all three PSI antibodies. Antibodies raised to PsbA cross-reacted to a diffuse band of $\approx$32 kDa in all three samples with

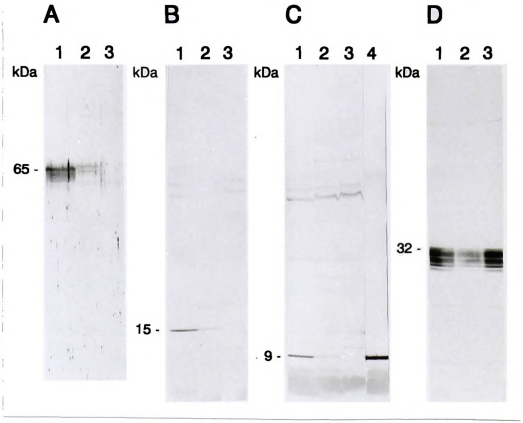

Figure 3.5. Immunoblots of thylakoid proteins from WT-MIXO, WT-LAHG, and ADK9. Lane 1 is WT-MIXO (5 μg chl); lane 2 is WT-LAHG (1.5 μg chl); lane 3 is ADK9 (0.75 μg chl); lane 4 (panel C) is purified PSI complex from wild-type *Synechocystis* 6803 (5 μg chl). Immunoblots were probed with PsaA/B antibody (panel A), PsaD antibody (panel B), PsaC antibody (panel C), and PsbA antibody (panel D). Relative molecular weights were estimated by comparison with prestained molecular size standards (Diversified Biotech) and are expressed in kDa.

approximately equal intensity.

DISCUSSION

Synechocystis 6803 as a genetic system

Synechocystis 6803 serves as an excellent system for the genetic alteration of photosynthetic proteins. Its ease of transformation, active homologous recombination, and ability to express bacterial drug resistance genes simplify targeted mutagenesis, providing that proper conditions for selection are met. Since *Synechocystis* 6803 maintains multiple copies of its genome per cell, complete segregation of a mutation may only be accomplished if the presence of wild-type copies provides no selective advantage to the cell. In order to mutagenize the core polypeptides of PSI, heterotrophic growth conditions (LAHG) for *Synechocystis* 6803 were developed by Anderson and McIntosh (Anderson and McIntosh, 1991). Procedures for isolation of PSI and PSII complexes (Rögner *et al.,* 1990) and well-characterized PSI and PSII EPR signals (Barry and Babcock, 1987; Golbeck and Bryant, 1991) make analysis of mutants relatively straightforward.

Response of wild-type cells to LAHG conditions

Wild-type *Synechocystis* 6803 grown under LAHG conditions has dramatically less chlorophyll per cell, when compared to WT-MIXO. When PSII activity and EPR signal II intensity are expressed per cell, the amount of

active PSII in WT-LAHG was only slightly less than in WT-MIXO. The signal intensity from PsbA antibody was approximately equal in WT-MIXO and WT-LAHG, while the signals from PSI antibodies were lower in WT-LAHG compared with WT-MIXO. The ratio of PSI to PSII, reflected by the relative intensity of EPR signal I to signal II, was greater for WT-MIXO than for WT-LAHG; on the order of 8 - 10:1 for WT-MIXO and 2 - 3:1 for WT-LAHG (Figure 3.4, panels C and D). A similar ratio of PSI to PSII for light-grown cells (9:1) was determined by (Rögner *et al.,* 1990). Thus, the reduction in chlorophyll in WT-LAHG cells was due primarily to a reduction in the amount of PSI, while the amount of PSII remained approximately the same. However, the pool of *psaA-psaB* transcript drops only 20% when *Synechocystis* 6803 is grown under LAHG conditions, while the pool of *psbA* message drops >70% (Smart and McIntosh, 1991, see Chapter 2). This indicates that there is some form of post-transcriptional regulation of PSI and PSII accumulation in cells grown under LAHG conditions.

Inactivation of *psaA*

We have used LAHG conditions for the selection of a *psaA*-inactivation mutant of *Synechocystis* 6803. This mutant, ADK9, contains no detectable functional PSI. Signal I, from P_{700}^{+}, was not present in the EPR spectrum of ADK9. Antibodies raised to the P_{700}-apoproteins from spinach, which cross-reacted to protein from wild-type *Synechocystis* 6803, did not detect the P_{700}-apoproteins in ADK9 thylakoids. Upon probing a northern blot of ADK9

RNA with the *psaA* gene, only a truncated *psaA-psaB* transcript, ≈1.2 kb in length was detected (5.0 kb in wild-type, Figure 3.2B). This is too short to encode the full length *psaA* or *psaB*. ADK9 displayed greatly reduced rates of oxygen evolution with no artificial acceptor. The low rate of oxygen evolution that was observed in whole ADK9 cells may simply represent saturation of the quinone pool, or it may indicate there was an undefined electron acceptor from PSII. This activity was fully inhibited by DCMU, an inhibitor of PSII (data not shown). Three possible electron acceptors for PSII in ADK9 are: ferredoxin, which reduces $NADP^+$, as proposed by (Arnon *et al.,* 1981); cytochrome oxidase *via* the cytochrome b_6/f complex, which is shared between photosynthesis and respiration in cyanobacteria (Sandmann *et al.,* 1984); or a hydrogenase, which would reduce H^+ to H_2 gas and has been purified from cyanobacteria (Ewert and Smith, 1989). The amount of chlorophyll per cell was greatly reduced in ADK9, allowing the turquoise-blue color of the phycobilisomes to predominate. This would be expected with the loss of the PSI core polypeptides, which bind over 100 chlorophyll molecules per P_{700} (Lundell *et al.,* 1985). By inspection of cells on plates illuminated with long-wave UV light (366 nm), fluorescence yield from ADK9 was greater than from WT-LAHG or WT-MIXO, a classic phenotype of mutants impaired in photosynthesis (Figure 3.6) (Girard *et al.,* 1980; Miles *et al.,* 1979).

Antibodies raised to PsaC and to PsaD, membrane-extrinsic proteins of

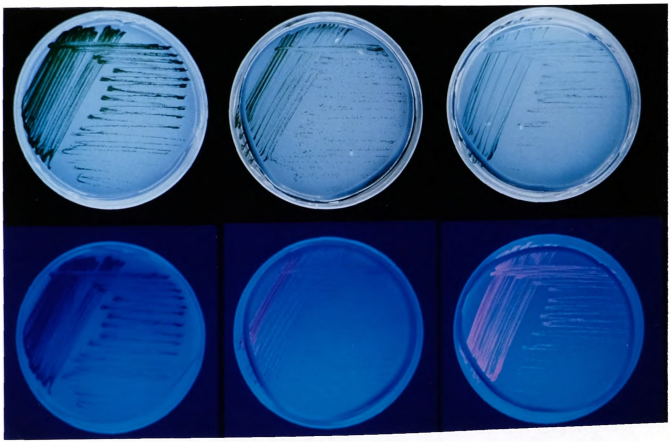

Figure 3.6. Petri dishes with WT-MIXO, WT-LAHG, and ADK9. Photographs were taken of one Petri dish each of WT-MIXO (left), WT-LAHG (center), and ADK9 (right). Photos in the top row were taken under illumination by white light; photos in the bottom row were taken under ultraviolet light (366 nm).

PSI, which cross-reacted to protein in wild-type thylakoids failed to detect those proteins in ADK9 thylakoids. This suggests that PsaC and PsaD do not accumulate in the absence of P_{700}-apoproteins. Despite the absence of the PSI core in ADK9, PSII assembled and formed functional complexes. The EPR spectrum from ADK9 included signal II, from Y_D^+, a tyrosine radical in the D2 polypeptide. Adding DCBQ, an acceptor of PSII, stimulated oxygen evolution to near wild-type rates when expressed on a per cell basis. The D1 polypeptide accumulated in ADK9, since antibodies raised to D1 from *Amaranthus hybridus* cross-reacted to protein of the expected molecular mass (32 kDa) in ADK9 thylakoids. The loss of small PSI proteins concomitant with the inactivation of the PSI core, with the continued assembly of PSII, corresponds to previously characterized nuclear (Girard *et al.,* 1980; Chua *et al.,* 1975) and plastome (Girard-Bascou, 1987b) mutants of *C. reinhardtii* and a nuclear mutant of barley (Møller *et al.,* 1980). Genetic inactivation of PSI in strains with site-directed mutations in PSII may facilitate their analysis by removing contaminating signals from PSI.

Although ADK9 grew at wild-type rates under LAHG conditions, it did not divide under continuous illumination of 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The loss of PSI and the chlorophyll bound to it probably made ADK9 extremely sensitive to photoinhibition. We have not determined the maximum irradiance under which ADK9 will grow. Since ADK9 did evolve oxygen, although at a much lower rate, these cells may be able to grow autotrophically (in the light without glucose) by oxidizing water and passing those electrons to an as yet undefined

acceptor.

Conclusions

The use of LAHG conditions is sufficient for complete segregation of mutations in the PSI core in *Synechocystis* 6803. These conditions eliminate the selective advantage conferred by the presence of wild-type PSI proteins and avert the lethal effect of continuous light. The success of this inactivation of *psaA* is encouraging for planned site-directed mutagenesis in the study of structure/function relationships in PSI.

MATERIALS AND METHODS

Materials

All chemicals and reagents were of high purity and were obtained from Sigma Chemical Co. (St. Louis, MO) or Research Organics (Cleveland, OH). Enzymes were obtained from Gibco-BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA). Nitrocellulose was obtained from Schleicher and Schuell (Keene, NH). Radioactive nucleotide ([α-^{32}P]dATP) was obtained from Amersham (Arlington Heights, IL).

Strain and growth conditions

A glucose-tolerant (Williams, 1988), dark-growth-adapted (Anderson and McIntosh, 1991) strain of *Synechocystis* sp. PCC 6803 was grown in BG-11

medium supplemented with 5 mM *N*-tris[hydroxymethyl]-2-aminoethanesulfonic acid (TES) pH 8.0 and 5 mM glucose, as previously described (Jansson *et al.,* 1987). Mixotrophic cultures (WT-MIXO) were grown in continuous light (20 μmol·m^{-2}·s^{-1}), while heterotrophic cultures (WT-LAHG and ADK9) were grown under LAHG conditions, complete darkness, except 5 min light (40 μmol·m^{-2}·s^{-1}) every 24 h in the presence of 5 mM glucose (Anderson and McIntosh, 1991). For growth on solid medium, the above medium was supplemented with 1.5% w/v agar and 0.3% sodium thiosulfate as described (Anderson and McIntosh, 1991). When appropriate, media were supplemented with 5 μg/ml kanamycin sulfate. Cells used for membrane isolation were grown in 10 - 15 l of medium and were harvested using a Sorvall continuous flow rotor. Cell growth was measured by absorbance at 730 nm (OD_{730}) in a Gilford Response spectrophotometer using a glass, 10 mm pathlength cuvette. Cells were counted in a hemacytometer. Chlorophyll was extracted with methanol and quantified using described extinction coefficients (Lichtenthaler, 1987). Fluorescence emission was assessed qualitatively from cells on solid medium under illumination of long-wave UV light (366 nm).

Nucleic acid manipulations

All nucleic acid manipulations were performed using standard techniques (Sambrook *et al.,* 1989), except where otherwise noted. Genomic DNA was isolated from *Synechocystis* 6803 essentially as described (Williams, 1988), fractionated by agarose gel electrophoresis, and transferred to nitrocellulose

(Southern, 1975). RNA isolation and conditions for hybridizations were previously described (Smart and McIntosh, 1991, see Chapter 2). A 1.8 kb *Kpn* I fragment encoding most of *psaA* from *Synechocystis* 6803 (pLS18) (Smart and McIntosh, 1991, see Chapter 2) was random primer-labelled (Feinberg and Vogelstein, 1983) and used as a probe. Quantification of hybridization to Southern blots was performed using a Betascope machine (Betagen, Waltham, MA). Transformation of *Synechocystis* 6803 was performed essentially as described (Williams, 1988), except that cells were transferred to selective medium 30 and 60 h after initial plating on non-selective medium.

Oxygen evolution assays

Rates of oxygen evolution were measured using whole cells as previously described (Debus *et al.,* 1988). For wild-type cells, 10 μg chl·ml^{-1} was used; for ADK9 cells, 5 μg chl·ml^{-1} was used. Light (from projector lamps) passed through a copper sulfate solution and filtered with red cellophane provided saturating illumination. DCBQ (1 mM) was added when appropriate. Cells for oxygen evolution assays were grown to late exponential phase (OD_{730} = 0.6-1.0), harvested, and washed twice with BG-11.

Thylakoid membrane isolation

Cells for thylakoid membrane isolation were grown to late exponential phase, harvested by centrifugation, washed with BG-11, and resuspended in break buffer (0.8 M sucrose, 20 mM MES [2-[*N*-morpholino]-ethansulfonic

acid] pH 6.0, 1 mM EDTA [ethylenediaminetetraacetic acid], 2 mg/l DNase I, 3 mg/l PMSF [phenylmethylsulfonyl fluoride], 0.6 mg/l pepstatin a, 1.8 mg/l TPCK [*N*-tosyl-L-phenylalanine chloromethyl ketone]). The cells were broken in a Bead Beater (Biospec Products, Bartlesville, OK) essentially as described by Rögner *et al.* (Rögner *et al.,* 1990). Unbroken cells and cell debris were pelleted at 6000g for 15 min (Sorvall GSA rotor). The membranes were then precipitated with 25 mM calcium chloride and 5% w/v PEG-8000 (polyethylene glycol MW 8,000) and pelleted at 27,000g for 15 min (Sorvall GSA rotor). The membranes were resuspended in freeze buffer (25% v/v glycerol, 20 mM calcium chloride, 20 mM MES pH 6.0), pelleted at 48,000g for 30 min (Sorvall SS-34 rotor), then resuspended in freeze buffer and stored at -70°C. Purified PSI complex from wild-type *Synechocystis* 6803 was provided by J. Golbeck (Department of Biochemistry, University of Nebraska, Lincoln, NE). Protein concentration was determined using the assay of Lowry (Lowry *et al.,* 1951).

EPR spectra

Isolated thylakoid membranes were pelleted and resuspended to a final concentration of 0.28 - 1.7 mg chl·ml^{-1} in BG-11 / 16 μM DCMU / 0.25 mM EDTA. EPR spectra were obtained as previously described (Barry and Babcock, 1987). Illumination was provided by a high-intensity microscope light. Dark-adaptation was for at least 10 min. Conditions were as follows: power, 2 mW; field modulation, 0.4 mT; time constant, 200 msec; sweep time, 100 sec. Spectra were averaged from at least 5 scans. The gain used for dark-adapted

samples and for illuminated ADK9 was 8 x 10^6. The gain used for illuminated WT-MIXO was 1 x 10^6 and for illuminated WT-LAHG was 2 x 10^6.

SDS-PAGE and immunoblotting

Protein samples were separated by electrophoresis on a 10-17.5% gradient acrylamide resolving gel with a 5% stacking gel (Laemmli, 1970) (Laemmli, 1970). Samples were prepared as described Wynn *et al.*, (1989)(Wynn *et al.*, 1989). Gels were run at 25 mA constant current for 7-8 h. Protein was visualized using Coomassie stain or transferred to nitrocellulose overnight at 1 mA with cooling (Towbin *et al.*, 1979)(Towbin *et al.*, 1979). Relative molecular weights were estimated by comparison to prestained molecular size standards (Diversified Biotech, Newton Centre, MA). Immunoblots were incubated at least 1 h in blocking solution (10 mM Tris, pH 7.4; 0.9% sodium chloride (NaCl); 1% bovine serum albumin (BSA); 0.02% sodium azide (NaN_3); 2% dry milk), then incubated with the primary antibody (1:500 dilution) for 1-1.5 h at room temperature. After washing off the primary antibody (3 times for 15 min with 10 mM Tris, pH 7.4; 0.9% NaCl; 0.02% NaN_3; 0.1% Triton X-100; 0.05% SDS; 0.1% BSA) the blots were incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Kirkegaard and Perry, Gaithersburg,MD) (1:3000 dilution of a 0.5 mg/ml solution in block buffer). The detection reaction was performed in development buffer (100 mM Tris, pH 8.8; 100 mM NaCl; 5 mM magnesium chloride) using BCIP (5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt) and NBT (nitro blue

tetrazolium) as substrates (Knecht and Dimond, 1984). Antibodies raised in rabbit against the P_{700}-apoproteins (PsaA and PsaB) from spinach were the kind gift of Nathan Nelson (Roche Institute, Nutley, NJ). Antibodies raised to the PsaC protein from *Synechococcus* (Henry *et al.,* 1990) were the gift of James Guikema (Div. of Biology, Kansas State Univ., Manhattan, KS). Antibodies raised to the 32 kDa-D1 polypeptide from *A. hybridus* were previously described (Ohad *et al.,* 1985). PsaD antibodies, raised in rabbit to spinach protein, were the gift of Richard Malkin (Div. of Molecular Plant Biology, Univ. of California, Berkeley, CA).

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CHAPTER 4

Genetic inactivation of the *psaB* gene in *Synechocystis* sp. PCC 6803 disrupts assembly of photosystem I

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INTRODUCTION

The electron transfer components of PSI in plants, algae, and cyanobacteria appear to be bound by three polypeptides, PsaA, PsaB, and PsaC (Golbeck and Bryant, 1991). The PsaC protein has a molecular weight of approximately 9 kDa and is much like a ferredoxin with two [4Fe-4S] centers (Oh-oka *et al.,* 1988). The PsaA and PsaB proteins are homologous and form a heterodimer to bind the electron transfer components P_{700}, A_0, A_1, and F_X (Golbeck and Bryant, 1991). We are using directed mutagenesis to investigate the biogenesis of the PSI reaction center and to probe some of the critical structure/function relationships in the core polypeptides. The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 is well-suited for the genetic manipulation of PSI, because it is readily transformable, integrates DNA by homologous recombination, expresses bacterial drug-resistance genes, and may

be grown heterotrophically (Anderson and McIntosh, 1991; Williams, 1988). Targeted inactivation of the *psaA* gene in *Synechocystis* 6803 by selection and segregation under light-activated heterotrophic growth (LAHG) conditions has been described (Smart *et al.,* 1991, see Chapter 3). In addition, targeted inactivation of the *psaB* gene in the filamentous cyanobacterium *Anabaena* sp. ATCC 29413 has been reported (Toelge *et al.,* 1991).

This communication describes the targeted inactivation of the *psaB* gene and full characterization of the effect of that mutation on the biogenesis of the PSI reaction center.

RESULTS AND DISCUSSION

Fragments from plasmids pLS13 and pLS15 (Smart and McIntosh, 1991, see Chapter 2) were used in the construction of a plasmid (pLS1315K) in which the *Synechocystis* 6803 *psaB* gene is interrupted by a gene cassette conferring kanamycin resistance (Km^R). The insert region of pLS1315K, as well as a partial restriction map of the *Synechocystis* 6803 *psaA-psaB* operon, are shown in Figure 4.1. *Synechocystis* 6803 was transformed with pLS1315K and selection and segregation for Km^R colonies was performed under LAHG conditions, as previously described (Smart *et al.,* 1991, see Chapter 3). Since pLS1315K is not able to replicate in *Synechocystis* 6803, and this bacterium has an active homologous recombination system, Km^R colonies would most likely be the product of a gene replacement event, as depicted in Figure 4.1. This was confirmed by Southern analysis of genomic DNA from one of the Km^R colonies

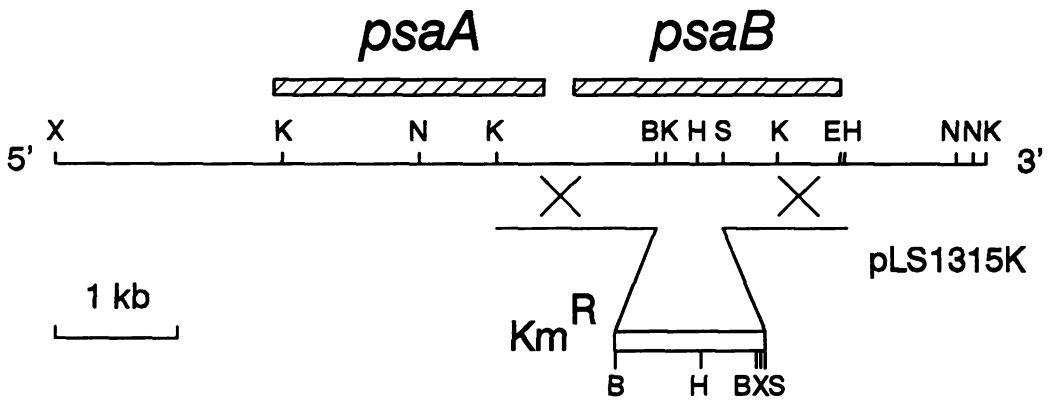

Figure 4.1. Partial restriction map of the *psaA-psaB* operon from *Synechocystis* 6803 and of plasmid pLS1315K. Boxes represent coding regions. The kanamycin resistance gene (Km^R) was taken from the plasmid pUC4K (Vieira and Messing, 1982; Taylor and Rose, 1988). Crosses represent possible regions of homologous recombination. Restriction sites are: X, *Xba* I; K, *Kpn* I; N, *Nco* I; B, *Bam*H I; H, *Hin*d III; S, *Sph* I; E, *Eco*R I.

(BDK8) that had been serially-streaked from single colonies at least five times (Figure 4.2A). RNA was isolated from BDK8 and subjected to northern analysis using a fragment of the *psaA* gene as a probe. A transcript of approximately 3.7 kilobases (kb) in length accumulates in BDK8 to wild-type levels and shows a similar pattern of degradation as wild type (Figure 4.2B). This transcript would be of sufficient length to encode all of PsaA and appears to terminate at approximately the position of the Km^R-cassette insertion.

The phenotype of BDK8 indicates that there are major changes in the composition of the thylakoids of this mutant. There is a decrease in the chlorophyll per cell (Table 4.1), allowing the turquoise blue color of the phycobilisomes to predominate (not shown). Likewise, there is a lower proportion of chlorophyll to protein in the membranes of BDK8 (Table 4.1). The BDK8 mutant is sensitive to continuous light above ≈ 3 $\mu mol \cdot m^{-2} \cdot s^{-1}$. BDK8 cells show very low rates of oxygen evolution in the absence of an artificial electron acceptor (Table 4.1). However, in the presence of 2,6-dichlorobenzoquinone (DCBQ), an artificial acceptor of electrons from PSII, rates of oxygen evolution are wild type, when expressed per cell. Electron paramagnetic resonance (EPR) spectroscopy revealed a dark-stable feature of approximately wild-type intensity with a linewidth of 2 milliTesla (mT) centered at g=2.0043, typical of signal II from tyrosine Y_D^+ in PSII (Figure 4.3). Signal I, from P_{700}^+ in PSI, is absent in the illuminated sample (Figure 4.3). The PSI proteins PsaA, PsaB, PsaC, and PsaD were undetectable by western analysis of BDK8 membranes (Figure 4.4A,B,C). However, the PSII reaction center

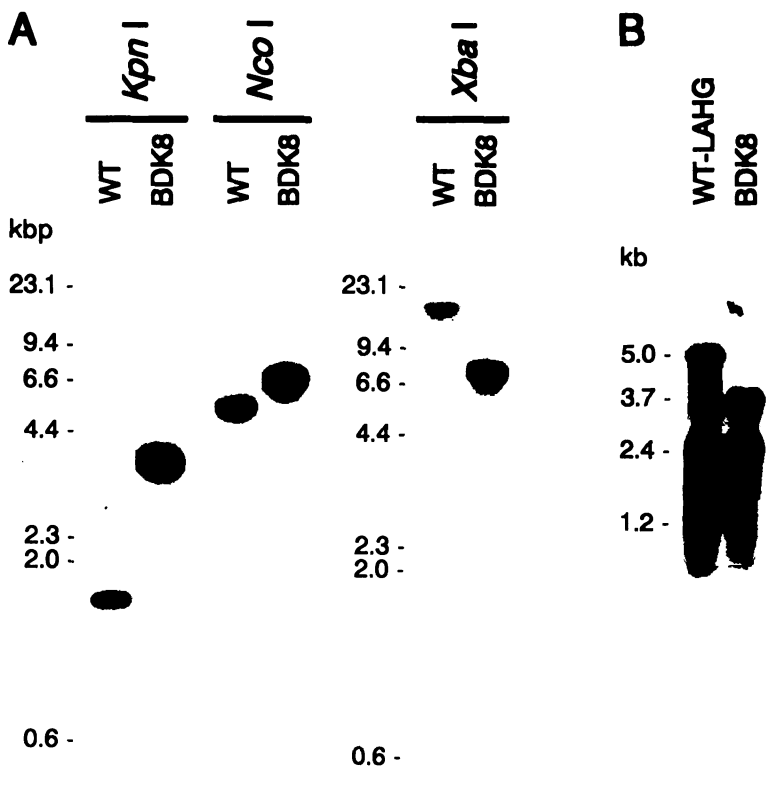

Figure 4.2. Autoradiographs of Southern and northern blots of wild type and BDK8. **A.** Southern blot of genomic DNA from wild type (WT) and BDK8 digested with the restriction enzymes indicated and probed with a 1.4-kb *Kpn* I fragment encoding the 3'-portion of *psaA* and the 5'-portion of *psaB*. The *Xba* I digests were run on a separate gel. 5 μg of DNA was loaded in each lane. **B.** Northern blot of RNA from LAHG-grown wild type cells and from BDK8, separated using an agarose-formaldehyde gel and probed with a 1.8-kb *Kpn* I fragment encoding most of *psaA*. Size markers were ethidium bromide-stained fragments from an RNA ladder (Gibco-BRL). DNA and RNA were isolated as in Smart and McIntosh (1991, see Chapter 2).

Table 4.1. Chlorophyll determinations and O_2 evolution assays[a].

	WT-LAHG	BDK8
Chlorophyll quantification		
Whole cells		
μg chl/(ml·OD_{730})	0.81±0.31	0.55±0.11
Membranes		
μg chl/mg protein	9.9±0.4	7.3±0.4
Oxygen evolution		
1 mM DCBQ		
μmol O_2/(mg chl·h)	832±194	1166±285
μmol O_2/(l·OD_{730}·h)	674	641
no added acceptor		
μmol O_2/(mg chl·h)	537±79	236±65
μmol O_2/(l·OD_{730}·h)	435	130

[a]WT-LAHG data taken from and assays performed as in Smart *et al.* (1991, see Chapter 3).

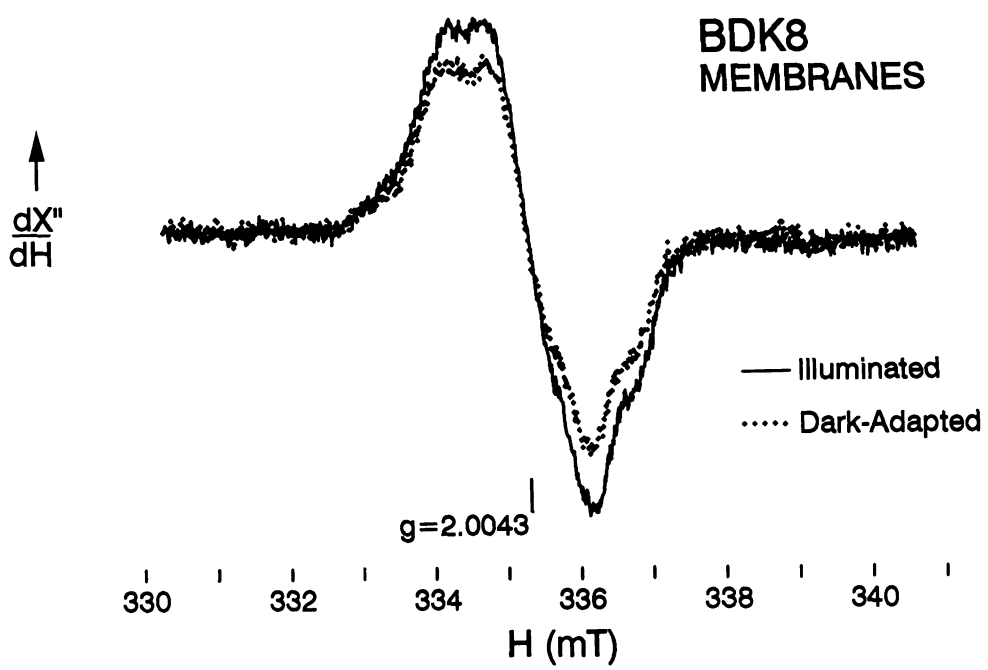

Figure 4.3. Room temperature EPR spectra of BDK8 membranes. Solid line is the spectrum taken while illuminating the cavity with a high-intensity microscope lamp. Dashed line is the spectrum taken after dark-adaptation. Spectrometer conditions were: power, 20 mW; field modulation, 4G; time constant, 200 ms; sweep time, 200 s; gain, 2.5 x 10^6; sample concentration, 0.6 mg chl/ml.

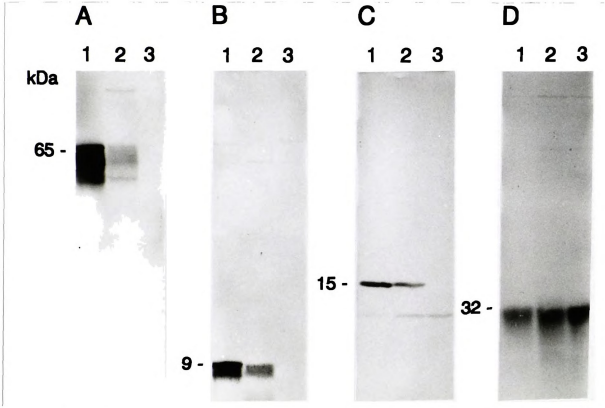

Figure 4.4. Immunoblots of WT-MIXO, WT-LAHG, and BDK8 membrane proteins. Samples were isolated from light-grown wild type (WT-MIXO) (1), LAHG-grown wild type (WT-LAHG) (2), and BDK8 (3). Blots were probed with antibodies recognizing PsaA/B (panel A), PsaC (panel B), PsaD (panel C), and PsbA (panel D). Equal amounts of protein (150 μg) were loaded in each lane. For panels A and D, proteins were separated using 10% SDS-PAGE; for panels B and C, 17% SDS-PAGE was used. Membranes were isolated and immunoblotting was performed as described in Smart *et al.* (1991, see Chapter 3).

protein PsbA was detected at near wild-type levels on immunoblots of BDK8 membranes (Figure 4.4D).

The *psaA* and *psaB* genes encode homologous proteins, suggesting that one arose by duplication of the other (Fish *et al.,* 1985). In particular, residues that may be important for the dimerization of the reaction center are conserved between the two polypeptides. These are: two conserved cysteine residues in each of the polypeptides that probably serve as ligands to the [4Fe-4S] center F_X (Golbeck and Bryant, 1991) and a series of conserved leucines spaced seven residues apart in each protein, which may form a leucine zipper (Kössel *et al.,* 1990; Webber and Malkin, 1990). Thus, the possibility exists that homodimers of either PsaA or PsaB may form. The insertional mutation in BDK8 blocked expression of the *psaB* gene, but a stable transcript of sufficient length to encode PsaA did accumulate to near wild-type levels. However, no PsaA protein could be detected by immunoblotting, and no functional P_{700} was detected by EPR spectroscopy. These data strongly suggest that stable PsaA homodimers do not form.

The phenotype of BDK8, a mutant in *psaB*, is nearly identical to that of ADK9, a mutant with an insertional mutation in *psaA* that also blocks expression of *psaB* (Smart *et al.,* 1991, see Chapter 3). Both mutations block stable assembly of PSI, but retain near wild-type levels of functional PSII. A similar phenotype was observed in a mutant of *Anabaena variabilis* ATCC 29413 in which *psaB* was insertionally inactivated (Toelge *et al.,* 1991) and in chemically-generated mutants of *psaB* in *Chlamydomonas reinhardtii*

(Girard-Bascou, 1987). The strain BDK8 is also extremely light-sensitive, like ADK9, probably due to lethal photo-inhibition of PSII, left unprotected by the loss of all of the PSI chlorophyll. However, BDK8 can grow in very dim light ($\approx$3 μmol·m^{-2}·s^{-1}) in the presence of glucose. We have used the genetic inactivation of PSI to an advantage in the study of site-directed mutations of PSII, since the biochemical purification of PSII complex is greatly simplified in mutants lacking PSI. The development of PSI$^-$ recipient strains for the mutagenesis of *psbA* and *psbD* will be described elsewhere (Smart *et al.*, submitted; see Chapter 5).

The ability to recover mutants with *psaB* genetically inactivated indicates that site-directed mutagenesis of this gene is possible. We have initiated experiments to mutagenize residues in PsaB thought to play roles in coordinating electron transport components and/or in reaction center dimerization and structure.

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CHAPTER 5

Genetic manipulation of the cyanobacterium *Synechocystis* sp. PCC 6803: development of strains lacking PSI for the analysis of mutations in PSII

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INTRODUCTION

The study of structure and function relationships in the PSII reaction center has been aided in recent years by the resolution of the three-dimensional structure of the bacterial reaction center (Deisenhofer and Michel, 1989; Allen *et al.,* 1987). The conservation of overall structural and functional components has enabled accurate predictions of the corresponding architecture of PSII (Michel and Deisenhofer, 1988). However, the non-oxygen-evolving bacterial reaction center provides few clues about the organization of the oxygen evolving complex (OEC) of PSII and the mechanism of water oxidation. In contrast, a wealth of biochemical, biophysical, and physiological data have been collected describing the enzymatic and chemical properties of the four Mn cluster that forms the active site of the OEC (Diner and Joliot, 1977;

Ghanotakis and Yocum, 1990). This Mn cluster serves to accumulate the oxidizing equivalents needed for the oxidation of water in a linear series of S-states driven by four single-photon events (Kok *et al.,* 1970; Forbush *et al.,* 1971). By using molecular genetics, precise determination of the amino acids necessary for the coordination of this unique and crucial structure may be possible.

Study of the structure and function of the photosystems has been accelerated by the use of the transformable unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Debus *et al.,* 1988a; Vermaas *et al.,* 1988a). This strain is naturally competent and has an active homologous recombination system, making it very amenable to molecular genetic manipulation (Williams, 1988). *Synechocystis* 6803 may also be grown photoheterotrophically in the presence of DCMU and glucose (Williams, 1988). Photoheterotrophic growth conditions negate the selective advantage conferred by the presence of wild-type PSII and have allowed the segregation and recovery of directed mutations in PSII reaction center genes (Debus *et al.,* 1988a; Metz *et al.,* 1989; Nixon and Diner, 1992; Vermaas *et al.,* 1988a).

Synechocystis 6803 lacks a trait found in chloroplast thylakoid membranes, which allows for easy purification of PSII: lateral heterogeneity of PSI and PSII (Andersson and Anderson, 1980). In chloroplasts, PSII is concentrated in the stacked membranes, while PSI is confined to the unstacked regions (Andersson and Anderson, 1980). Published protocols for the purification of PSII from *Synechocystis* 6803 rely on selective detergent

solubilization, sucrose density centrifugation, and/or a series of chromatographic separations (Burnap *et al.,* 1989; Noren *et al.,* 1991; Rögner *et al.,* 1990), techniques that can reduce or destroy OEC activity, especially in mutants (N. Bowlby, unpublished results). Current techniques for the purification of PSII have served well for certain types of analysis of site-directed PSII mutants (Debus *et al.,* 1988a; Nixon and Diner, 1992) and detection of the EPR multiline spectrum from the S_2 state of the Mn cluster has been reported (Noren *et al.,* 1991). However, a method yielding highly purified and active PSII with a minimum of biochemical manipulation would be highly beneficial for the analysis of mutants in the OEC.

To facilitate such studies, we have embarked on a genetic approach to the elimination of PSI, a detrimental contaminant of PSII preparations, in *Synechocystis* 6803. Since there is no effective inhibitor of PSI activity, cells must be grown heterotrophically to eliminate the selective advantage conferred by wild-type PSI. *Synechocystis* 6803 may be grown in complete darkness using glucose as a carbon source, requiring only a brief (5 min) pulse of light every 24 h (Anderson and McIntosh, 1991). By selecting under these light-activated heterotrophic growth (LAHG) conditions (Anderson and McIntosh, 1991), we have successfully inactivated the genes encoding the reaction center proteins of PSI, *psaA* and *psaB* (Smart *et al.,* 1991, see Chapter 3; Smart and McIntosh, 1992, see Chapter 4). These mutants were devoid of any detectable PSI complex, but still accumulated functional PSII at wild-type levels.

Here we describe the development of recipient strains for the

mutagenesis of the PSII reaction center genes *psbA* and *psbD*. These strains, D1$^-$/PSI$^-$ and D2$^-$/PSI$^-$ have mutations in *psaA* that prevent assembly of PSI. In order to avoid problems associated with the segregation of a mixture of wild-type and mutant *psbA* or *psbD* sequences, the regions of the chromosome targeted for mutagenesis have been deleted. In *Synechocystis* 6803, *psbA* is present in three copies (Jansson *et al.*, 1987), therefore, deletions were introduced into all three copies by replacing those regions of the chromosome with three different drug-resistance cassettes (I. Sithole, N.R. Bowlby, J. Sinclair, G.T. Babcock, L. McIntosh, in preparation). Likewise, *psbD* is present in two copies (Williams and Chisholm, 1987), into both of which deletions were introduced. The rationales for attempting particular mutations in the *psbA* or *psbD* genes will be discussed elsewhere (Sithole *et al.*, in preparation). Characterization of the strains D1$^-$/PSI$^-$ and D2$^-$/PSI$^-$ also gives us insight into the biogenesis of PSII and aspects of cyanobacterial physiology.

MATERIALS AND METHODS

Materials

Chemicals and antibiotics used were of high purity and were obtained from Sigma Chemical Co. (St. Louis, MO) or Research Organics (Cleveland, OH). Restriction and other enzymes were purchased from New England Biolabs (Beverly, MA). Radioactive isotope ([α-^{32}P]dATP) was purchased from Amersham (Arlington Heights, IL), nitrocellulose membrane from Schleicher and Schuell (Keene, NH), and bacto-agar from Difco (Detroit, MI).

Strains and growth conditions

All studies were performed using a glucose-tolerant strain of the cyanobacterium *Synechocystis* sp. PCC 6803 (Williams, 1988). The mutant ADK9, an insertional inactivation mutant of *psaA*, was previously described (Smart *et al.,* 1991, see Chapter 3). The mutant 3Δ*psbA*, with all three copies of *psbA* deleted, will be described elsewhere (Sithole *et al.,* in preparation). Cells were grown in liquid BG-11 medium with 5 mM glucose or on solid BG-11 with 5 mM glucose and 1.5% purified Bacto-Agar under LAHG conditions, as previously described (Anderson and McIntosh, 1991). Transformation of *Synechocystis* 6803 was performed essentially as described (Williams, 1988). For analysis of mutants, cells were grown in carboys containing 15 l of medium, were harvested using a continuous flow rotor (DuPont Sorvall, Wilmington, DE), and were frozen at -70°C in BG-11 with 15% v/v glycerol. When appropriate, media were supplemented with antibiotics in the following concentrations: chloramphenicol, 10 mg/l; erythromycin, 5 mg/l; spectinomycin, 20 mg/l; kanamycin, 5 mg/l; and gentamycin, 1 mg/l.

Nucleic acids

All nucleic acid manipulations were performed using standard procedures (Sambrook *et al.,* 1989), unless otherwise stated. DNA was isolated from *Synechocystis* 6803 using a modification of the procedure described by Golden *et al.* (1987) for the isolation of RNA from *Synechocystis* 6803. The aqueous phase obtained after vortexing the cells with glass beads, buffer,

phenol:chloroform, and detergents was extracted twice with chloroform, then purified over a cesium chloride gradient with ethidium bromide by centrifugation in a Beckman vTi50 rotor at 45,000 R.P.M. for 18 h. The ethidium-bromide-stained DNA band was recovered, extracted with isopropanol to remove ethidium bromide, and ethanol precipitated. Hybridization conditions and preparation of radiolabeled probes were previously described (Smart and McIntosh, 1991, see Chapter 2). The probe for *psbD2* was a 0.5-kb *Kpn* I/*Sma* I fragment from plasmid pRD655 (Debus *et al.,* 1988a). The *psbD1* probe was a 3.5-kb *Hin*d III fragment from plasmid pRD1219 (Debus *et al.,* 1988a). A 1.8-kb *Kpn* I fragment from pLS18 (Smart and McIntosh, 1991, see Chapter 2) was used to probe for *psaA*.

Chl and protein analysis

Chl was extracted from whole cells or from membranes using methanol and was quantified using the extinction coefficients of Lichtenthaler (1987). Cells were broken using a bead beater (Biospec Products, Bartelsville, OK), and membranes were prepared essentially as previously described (Smart *et al.,* 1991, see Chapter 3). Membrane proteins were prepared for electrophoresis as described by Wynn *et al.* (1989) and were separated by 10% SDS-PAGE using the buffers of Laemmli (1970). Proteins were transferred to nitrocellulose and immunoblotting was performed as described by Towbin *et al.* (1979) and Smart *et al.* (1991, see Chapter 3), respectively. Antibodies raised to the PsaA/B proteins from *Synechococcus* have been described (Henry *et al.,* 1990), as have

antibodies raised to a portion of the D1 protein from *Amaranthus hybridus* (Ohad *et al.,* 1985). Antibodies raised to the D2 polypeptide from spinach were previously described (Vermaas *et al.,* 1988b).

EPR spectroscopy

EPR spectroscopy was performed at room temperature using a Bruker ER200D spectrometer as previously described (Smart *et al.,* 1991, see Chapter 3). Spectrometer conditions were: power, 20 mW; modulation amplitude, 0.4 mT; time constant, 200 ms; and sweep time, 200 s. Illumination was provided by a high-intensity microscope lamp. Dark-adaptation was for at least 10 min.

RESULTS

Plasmid constructs and transformations

We used different strategies to create the two recipient strains for mutagenesis of *psbA* and *psbD.* In the case of the D2-recipient, we started with a PSI$^-$ mutant, ADK9 (Smart *et al.,* 1991, see Chapter 3), and sequentially introduced deletions into both copies of *psbD.* To create the D1-recipient strain, we inactivated the *psaA* gene in a strain, 3Δ*psbA* (Sithole *et al.*, in preparation), which had deletions in all three copies of *psbA.*

The first step in constructing the D2-recipient was the deletion of the *psbD2* gene. The strain ADK9 was transformed with the plasmid pRD655Cm^R (Debus *et al.,* 1988a), in which most of the *psbD2* gene had been replaced by a

gene encoding resistance to chloramphenicol. Colonies resistant to kanamycin and chloramphenicol were selected and streak-purified from single colonies to accomplish gene segregation. DNA was isolated from one of these colonies, ADK9Δ*psbD2*, and was subjected to Southern analysis using the 3'-portion of the *psbD2* gene as a probe (Figure 5.1A). Since these cells were found to be segregated for the deletion of *psbD2* (Figure 5.1A), they were then transformed with the plasmid pSLA1219:Gm^R (Figure 5.2A). This plasmid was a modification of pRD1219 (Debus *et al.,* 1988a). In pSLA1219:Gm^R most of the *psbD1* gene had been replaced by a 2.0-kb *Bam*H I-fragment containing a gene encoding resistance to gentamycin (Yin *et al.,* 1988), originally purified from the plasmid pRZ1107, then subcloned into the *Bam*H I site of pUC119 (Vieira and Messing, 1987), yielding pUC119-gen. Colonies resistant to chloramphenicol, kanamycin, and gentamycin were selected and steak-purified from single colonies to allow for segregation. DNA was isolated from one of these colonies to assess whether there had been segregation of the *psbD1* deletion by Southern analysis (Figure 5.1B). This strain was designated $D2^-/PSI^-$.

The strain 3Δ*psbA* was generated by targeted deletion of *psbA2* in a strain with deletions in *psbA1* and *psbA3* (Debus *et al.,* 1988b), resulting in a strain with genes encoding resistance to chloramphenicol, spectinomycin, and erythromycin in the chromosome (Sithole *et al.*, in preparation). In order to inactivate PSI genetically in this strain, we constructed a plasmid, pLS1813G, with a portion of the *psaA* gene replaced by a *Bam*H I-*Kpn* I fragment from the plasmid pUC119-gen encoding resistance to gentamycin (Figure 5.2B). The

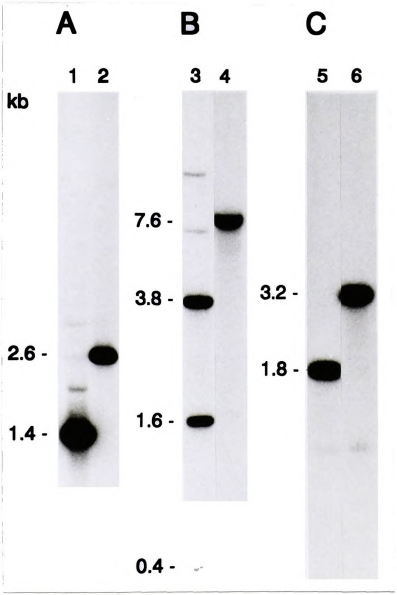

Figure 5.1. Autoradiographs of Southern blots of genomic DNA from wild-type and mutant cells. Lanes are: 1, 3, 5, wild type; 2, ADK9Δ*psbD2*; 4, D2^{-}/PSI^{-}; 6, D1^{-}/PSI^{-}; 5 μg DNA per lane. Panels are: A, *psbD2* probe, DNA digested with *Hinc* II; B, *psbD1* probe, DNA digested with *Bst*E II; C, *psaA* probe, DNA digested with *Kpn* I. Autoradiographs were exposed for different lengths of time.

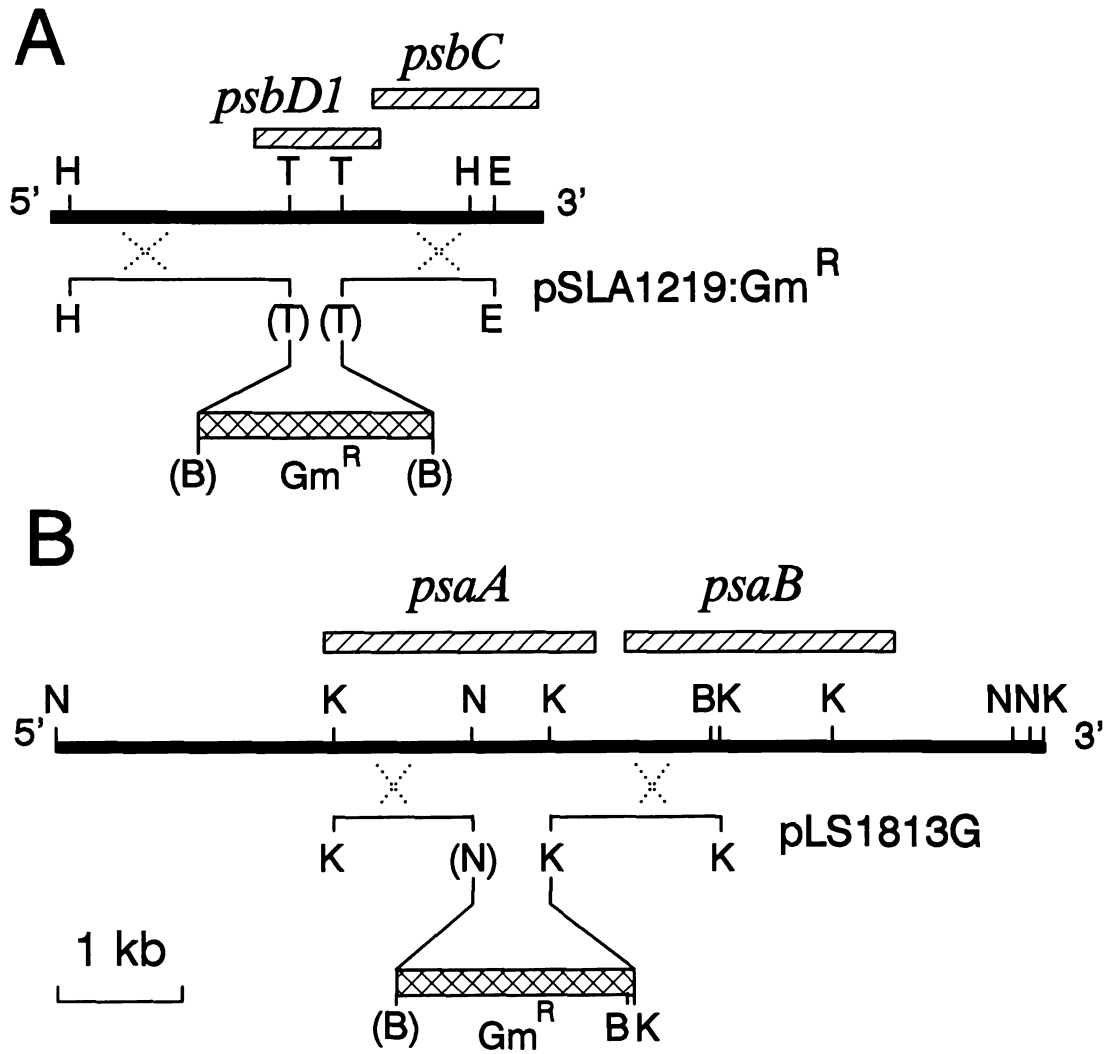

Figure 5.2. Partial restriction maps of plasmids used for mutagenesis and of portions of the *Synechocystis* 6803 chromosome. **A.** Map of the *psbD1-psbC* region and of the insert region from the plasmid pSLA1219:Gm^R. **B.** Map of the *psaA-psaB* operon and of the insert region from the plasmid pLS1813G. Thick lines represent regions of the *Synechocystis* 6803 chromosome. Hatched boxes represent coding regions. The cross-hatched boxes represent the gentamycin resistance gene cassette. Crosses depict possible sites of homologous recombination. Restriction sites in parentheses were destroyed in the cloning process. Restriction sites are represented by: H, *Hin*d III; T, *Bst*E II; E, *Eco*R I; B, *Bam*H I; N, *Nco* I; K, *Kpn* I.

strain 3Δ*psbA* was transformed with pLS1813G and colonies resistant to chloramphenicol, erythromycin, spectinomycin, and gentamycin were selected and streak-purified from single colonies to accomplish segregation. DNA was isolated from one of the resistant colonies and was subjected to Southern analysis to assess whether there had been segregation of the *psaA* mutation (Figure 5.1C). This strain was designated D1$^-$/PSI$^-$.

Characterization of D1$^-$/PSI$^-$ and D2$^-$/PSI$^-$

Analysis of the strains D1$^-$/PSI$^-$ and D2$^-$/PSI$^-$ included chl determinations, immunoblotting, and EPR spectroscopy. Chl determinations performed on whole cells revealed very low levels of chl per cell in strain D2$^-$/PSI$^-$, relative to WT-MIXO (wild-type cells grown under mixotrophic conditions, light in the presence of glucose) or WT-LAHG (wild-type cells grown under LAHG conditions) (Table 5.1). The chl levels in strain D1$^-$/PSI$^-$ were also lower than in WT-LAHG (Table 5.1), but greater than in D2$^-$/PSI$^-$. The ratio of chl to protein in isolated membranes follows the same trend (Table 5.1). Both strains, D1$^-$/PSI$^-$ and D2$^-$/PSI$^-$, grew at near wild-type rates under LAHG conditions, but did not grow in continuous light above ≈3 μmol·m^{-2}·s^{-1} (data not shown). Also, both cell types exhibited the turquoise-blue color seen previously in cells lacking PSI (Smart *et al.,* 1991, see Chapter 3; Smart and McIntosh, 1992, see Chapter 4).

To determine if any PSI or PSII reaction center proteins were accumulating in the recipient strains, membrane proteins were subjected to

Table 5.1. Chlorophyll content of whole cells and thylakoid membranes.

	WT-MIXO*	WT-LAHG*	D1⁻/PSI⁻	D2⁻/PSI⁻
Whole cells				
μg chl/(ml·OD_{730})	3.66±0.63	0.81±0.31	0.31±0.06	0.15±N.D.
Membranes				
μg chl/mg protein	43.1±7.2	9.9±0.4	5.5±0.1	3.2±0.3

*Data taken from Smart *et al.* (1991, see Chapter 3).

N.D., not determined.

immunoblotting. Antibodies raised to the PsaA/B polypeptides did not detect the PSI reaction center proteins in membranes from either D1$^-$/PSI$^-$ or D2$^-$/PSI$^-$ (Figure 5.3A). Likewise, antibodies raised against D1 did not detect that protein in membranes from either of the two recipient strains, but did display apparent non-specific cross-reaction to a protein of ≈33 kDa (Figure 5.3B). However, when antibodies raised to the D2 polypeptide were used, a reduced amount of D2 protein was detected in membranes from the strain D1$^-$/PSI$^-$ (Figure 5.3C). Almost no D2 was detected in membranes from the strain D2$^-$ /PSI$^-$ (Figure 5.3C). Protein >55 kDa that was recognized by antibodies raised to D2 may represent dimers of D2 or may be protein exhibiting non-specific cross-reaction.

EPR spectroscopy is a sensitive and quantitative method for detecting redox components in the two photosystems. We collected room temperature EPR spectra from membranes of both recipient strains; conditions under which one would expect to detect both signal I (from P_{700}^+ in PSI) and signal II (from tyrosine Y_D^+ in PSII). The spectra from both recipient strains include a prominent feature centered at g=2.00425 with a linewidth of 1 mT and a lineshape and low intensity (Figure 5.4) that clearly indicate it does not arise from tyrosine Y_D^+ (g=2.0045, ΔH_{pp}≈2 mT) (Barry and Babcock, 1987). This feature is seen under illumination and in the dark and is of approximately equal intensity in samples from the two strains (Figure 5.4). There are no light-induced signals in the spectra from either recipient strain (Figure 5.4).

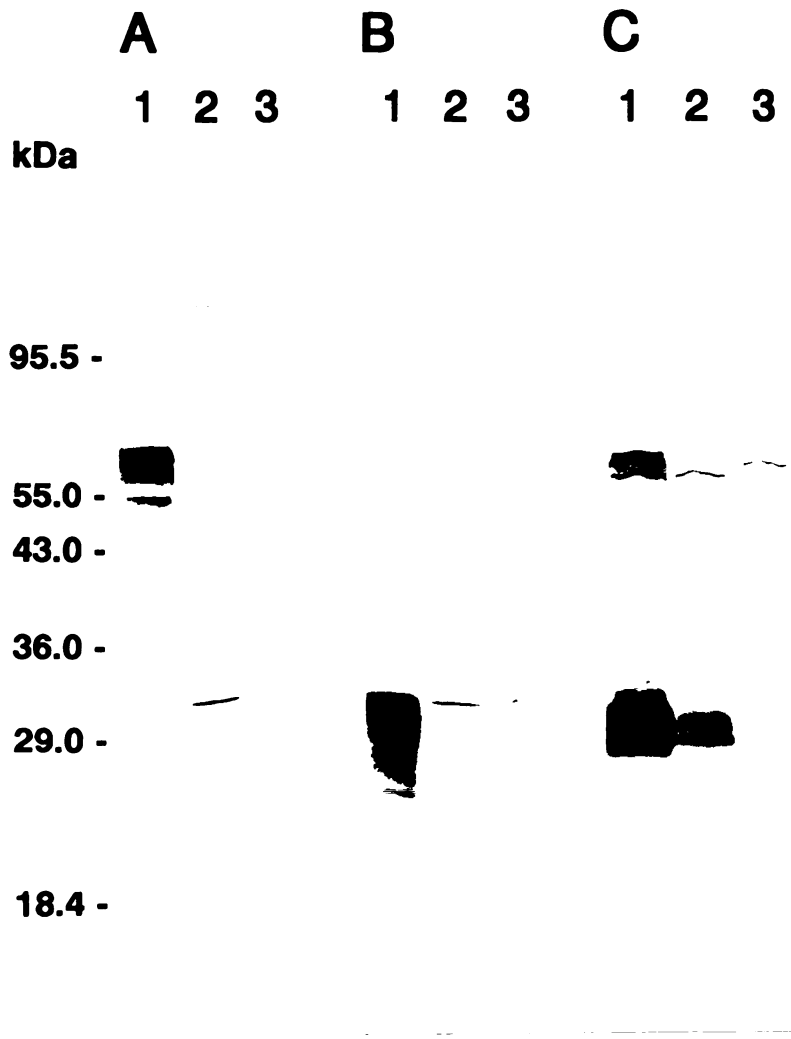

Figure 5.3. Immunoblots of membrane proteins isolated from wild type, D1$^-$/PSI$^-$, and D2$^-$/PSI$^-$. Lanes are: 1, wild type; 2, D1$^-$/PSI$^-$; 3, D2$^-$/PSI$^-$; 150 μg protein per lane. Antibodies used for the three panels recognized: A, PsaA/B; B, the D1 polypeptide; C, the D2 polypeptide. Molecular weight standards were prestained proteins purchased from Diversified Biotech (Newton Centre, MA).

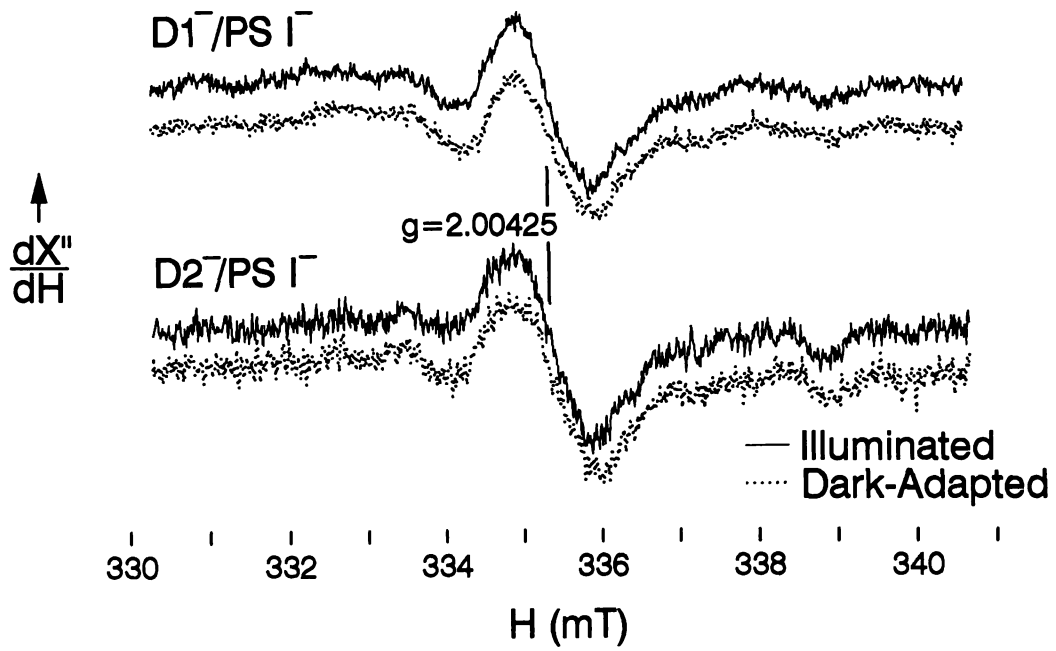

Figure 5.4. Room temperature EPR spectra of thylakoids from $D1^-/PSI^-$ and $D2^-/PSI^-$. The solid lines are the spectra obtained under constant illumination. The dotted lines are the spectra collected after dark-adaptation. Spectra were normalized to approximately equal chl concentrations. The magnetic field (H) is expressed in milliTesla (mT). The vertical position of each spectrum in the figure is unrelated to the scale of the vertical axis.

DISCUSSION

The purification of active PSII is essential for the analysis of site-directed mutations targeted at the OEC. The process of purification of PSII from *Synechocystis* 6803 is complicated by the lack of lateral heterogeneity of PSI from PSII that is found in chloroplasts (Andersson and Anderson, 1980). We have taken a genetic approach to eliminate PSI from mutants with site-directed changes in PSII, by generating recipient strains in which PSI has been genetically inactivated and deletions have been introduced into the targeted PSII reaction center genes. By deleting the regions targeted for mutagenesis, problems associated with segregating a mixture of wild-type and mutant sequences are avoided. This is the first published use of the gentamycin resistance gene (Yin *et al.,* 1988) for selection of mutations in a cyanobacterium. This gene adds another option for manipulation of the cyanobacterial chromosome. We plan to transform the strains D1$^-$/PSI$^-$ and D2$^-$/PSI$^-$ with plasmid DNA encoding *psbA2* or *psbD1*, respectively, including the desired site-directed mutation. The DNA used for the transformation will overlap the deleted regions and, since the plasmid vector cannot replicate in the cyanobacterium, will integrate into the *Synechocystis* 6803 chromosome by homologous recombination (Williams, 1988). An adjoining drug-resistance gene will be used to select for integration of the transforming DNA. This type of mutagenesis strategy allowing for rapid recovery of homozygous mutants has been described previously (Nixon and Diner, 1992).

Our previous analysis of isolated membranes from strains ADK9 or

BDK8 yielded EPR spectra with PSII signals of wild-type intensity and essentially free of other contaminating signals (Smart *et al.,* 1991, see Chapter 3; Smart and McIntosh, 1992, see Chapter 4). Thus, we are quite confident that the inactivation of PSI does not significantly alter the stable assembly of PSII in *Synechocystis* 6803. The strains D1$^-$/PSI$^-$ and D2$^-$/PSI$^-$ give us some insight into the biogenesis of PSII. Genetic inactivation of the two copies of *psbD*, which encode the D2 polypeptide, also prevented accumulation of the D1 polypeptide. However, targeted deletion of the three copies of *psbA*, which encode D1, caused only partial reduction of the accumulation of D2. The D2 protein that did accumulate in D1$^-$/PSI$^-$ did not assemble into functional PSII complex, since we were unable to detect EPR signal II or oxygen-evolving activity in the presence of DCBQ, an artificial electron acceptor from PSII (data not shown). For the most part, our observations concerning PSII biogenesis in PSI$^-$ strains correspond with those reported for strains containing PSI. When both copies of *psbD* were inactivated in a strain containing PSI, the D1 polypeptide and other PSII proteins failed to accumulate (Vermaas *et al.,* 1988b; Yu and Vermaas, 1990). In a mutant with the three copies of *psbA* inactivated, the proteins CP43, Cyt b_{559}, and the 33-kDa protein accumulated to levels only slightly lower than wild type, while the accumulation of the D2 and CP47 proteins was greatly reduced (Jansson *et al.,* 1987; Nilsson *et al.,* 1990). The difference in accumulation of D2 observed for strain D1$^-$/PSI$^-$ and a D1$^-$ strain containing PSI may be the result of different membrane isolation techniques or due to different antisera. Our results reaffirm previous

observations that both D1 and D2 are crucial for stable assembly and accumulation of PSII complex.

Assays of chl accumulation per cell serve well in estimating the accumulation of PSI or PSII reaction center proteins (Smart *et al.,* 1991, see Chapter 3; Smart and McIntosh, 1992, see Chapter 4) and may be used as a quick and easy assay for initial characterization of mutants. This would be expected, since cyanobacteria do not contain peripheral chl-binding antennae proteins. The chl content in the $D1^-/PSI^-$ cells is reduced relative to ADK9 or BDK8, cells lacking only PSI (Smart *et al.,* 1991, see Chapter 3; Smart and McIntosh, 1992, see Chapter 4). The chl content in $D2^-/PSI^-$ is about half that of $D1^-/PSI^-$. The higher chl levels in $D1^-/PSI^-$ may reflect the accumulation of low levels of D2. The low amount of chl in $D2^-/PSI^-$ may be bound to CP43 and CP47. We did not examine the accumulation of CP43 or CP47 in these strains. The chl levels in both strains $D1^-/PSI^-$ and $D2^-/PSI^-$ are higher than one would expect for cells that lack both PSI and the PSII reaction center. The elevated chl levels may be the result of free chl that is trapped in membranes or chl in some stage of synthesis or degradation, which may or may not be bound to protein.

We have shown previously that cells with mutations which inactivate the PSI reaction center genes exhibit normal assembly of stable PSII complex (Smart *et al.,* 1991, see Chapter 3; Smart and McIntosh, 1992, see Chapter 4). However, these strains are extremely light-sensitive, and, therefore, must be grown in very dim light ($\approx 3\ \mu mol \cdot m^{-2} \cdot s^{-1}$) or under LAHG conditions. The

genetic inactivation of PSII does not appear to have relieved that light-sensitivity, suggesting that there is some other light-induced component creating toxic elements or that respiration cannot function to synthesize ATP in light >3 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

The EPR spectra from D1$^-$/PSI$^-$ and D2$^-$/PSI$^-$ contained neither signal I, from P_{700}^+, nor signal II, from tyrosine Y_D^+ in D2. The prominent feature, common to spectra from both strains, was not light-induced. One possible explanation is that this feature may have arisen from a semi-quinone in the cytochrome b_6/f complex (G. T. Babcock, personal communication).

The strains D1$^-$/PSI$^-$ and D2$^-$/PSI$^-$ represent the first mutants in a photosynthetic organism with both photosystems genetically inactivated. As well as serving in the process of site-directed mutation of PSII genes, these strains may be particularly well-suited for other studies. In cyanobacteria, the Cyt b_6/f chain is shared between photosynthesis and respiration (Scherer, 1990). The coordinate regulation of electron flow between these pathways is poorly understood. These strains may be appropriate for the study of respiration in cyanobacteria, since the interaction with the photosystems has been eliminated. The membranes from D2$^-$/PSI$^-$ are essentially free of photosynthetic proteins, so they may serve as excellent starting material for the purification of the cytochrome b_6/f complex.

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CHAPTER 6

Mutational analysis of the structure and biogenesis of the photosystem I reaction center in the cyanobacterium *Synechocystis* sp. PCC 6803

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INTRODUCTION

The photosystem I (PSI) reaction center represents a grand complex of protein subunits, both membrane-bound and extrinsic, which assemble to bind cofactors that act to capture light energy and convert it into chemical energy (for recent reviews, see Golbeck (1992); Golbeck and Bryant (1991)). The coordination of the synthesis and assembly of the PSI subunits, which in plants and algae are encoded by both nuclear and plastome genes, may be regulated at many different levels. Our goal is to gain insight into the interaction of protein subunits that allows the formation of the protein scaffold, which binds electron transfer components, and the roles of those elements in the chemical reactions performed by PSI.

A heterodimer of homologous 82 - 83-kDa polypeptides, PsaA and PsaB,

forms the reaction center of PSI. The PsaA and/or PsaB polypeptides bind the chlorophyll (chl) *a* dimer P_{700}, and the electron acceptors A_0 (a chlorophyll *a*), A_1 (probably a phylloquinone (Golbeck and Bryant, 1991)), and F_X (a [4Fe-4S] center); as well as ≈100 antennae chl molecules (Golbeck, 1992). A third polypeptide, PsaC, binds the terminal electron acceptors F_A and F_B, both of which are [4Fe-4S] centers (Oh-oka *et al.,* 1988). There are at least eight additional polypeptides associated with PSI, some that may serve to facilitate binding of the soluble electron carriers plastocyanin and ferredoxin (Wynn and Malkin, 1988; Zilber and Malkin, 1988), and others with unknown functions. In plants and algae, the genes *psaA*, *psaB*, *psaC*, *psaI*, and *psaJ* are located in the plastome, while the genes encoding the other known PSI proteins are nuclear-encoded (Golbeck and Bryant, 1991).

Although attempts to determine the three-dimensional structure of PSI are progressing (Almog *et al.,* 1991; Witt *et al.,* 1988), the current resolution of the crystal structure does not allow one to define precisely the protein structures important for the assembly and function of the reaction center. In order to identify residues that may serve as important structural and/or functional components in the complex, we must rely on analysis of evolutionary conservation and on the current body of biochemical and biophysical data. The most convincing evidence for the roles of particular residues in the PSI reaction center concerns possible cysteine ligands to the [4Fe-4S] center F_X (Golbeck and Cornelius, 1986) and leucines lying in a conserved leucine zipper motif in the PsaA and PsaB proteins (Webber and Malkin, 1990; Kössel *et al.,* 1990).

Cysteine residues, with few exceptions, serve as the ligands to [4Fe-4S] centers (Palmer, 1973). There are only three conserved cysteine residues in PsaA and only two in PsaB. Two of the cysteines in PsaA are homologous to the PsaB cysteines, lying in a twelve-residue sequence that is conserved in all the deduced amino acid sequences of *psaA* and *psaB* genes sequenced thus far (Golbeck and Bryant, 1991). It has been proposed that F_X bridges the PsaA and PsaB polypeptides and is ligated by two cysteine residues from each of the reaction center proteins (Golbeck and Cornelius, 1986) (Figure 6.1). Immediately adjacent to the conserved cysteine motif in both PsaA and PsaB are series of conserved leucines, spaced seven amino acids apart and predicted to lie in α helices (Webber and Malkin, 1990; Kössel *et al.,* 1990) (Figure 6.1). The cysteine residues proposed to coordinate F_X lie in a position analogous to the DNA-binding region of bZIP proteins (Pu and Struhl, 1991; Webber and Malkin, 1990).

We are using site-directed mutagenesis to investigate the roles of the aforementioned cysteine and leucine residues in the biogenesis and function of the PSI reaction center (Figure 6.1). This genetic manipulation is most readily accomplished using the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, since it is easily transformed, has an active homologous recombination system, expresses bacterial drug resistance genes, and may be grown heterotrophically (Williams, 1988; Anderson and McIntosh, 1991). PSI in the cyanobacteria is very similar to that from higher plants, both in subunit composition and in gene organization (Ho and Krogmann, 1982; Golbeck and Bryant, 1991); thus,

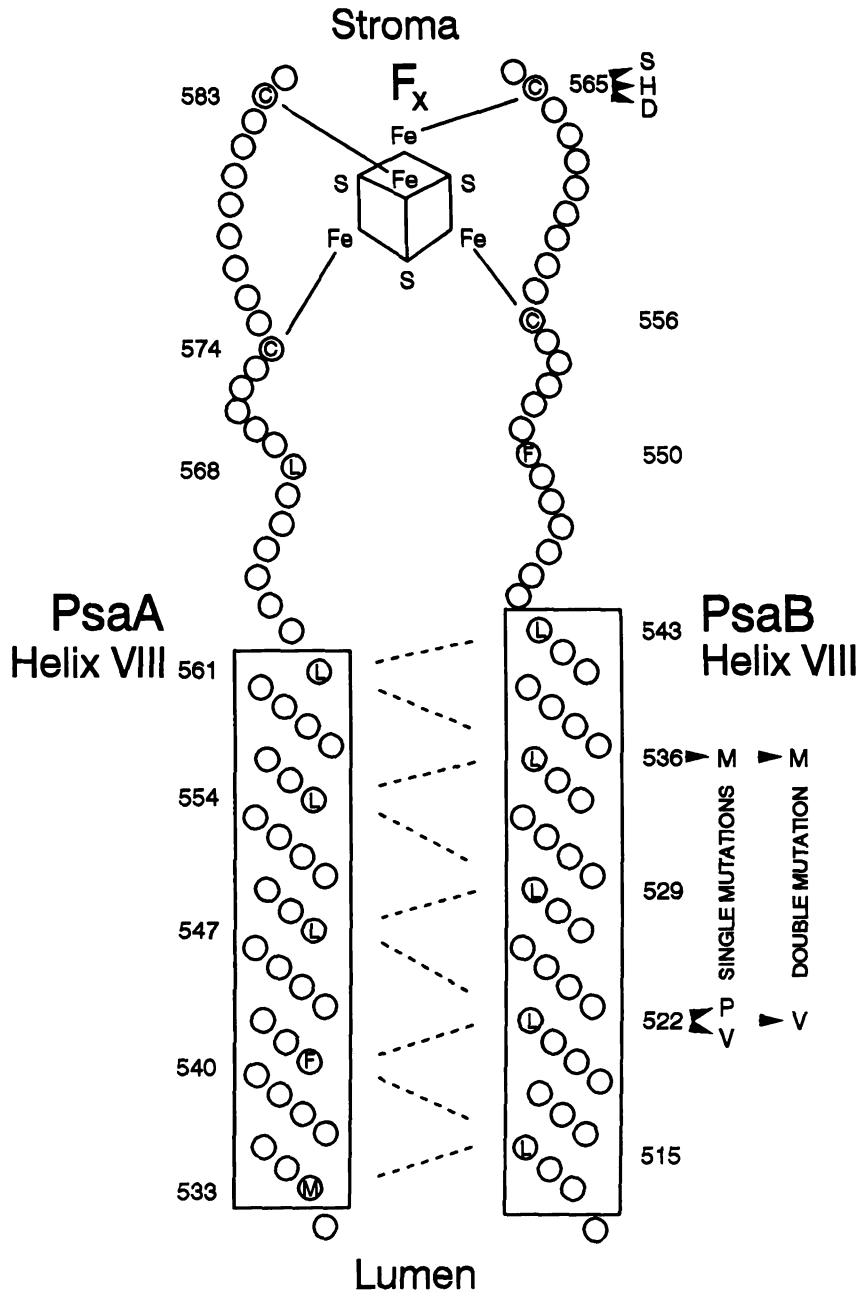

Figure 6.1. Cartoon of F_X and portions of PsaA and PsaB. Shown is a schematic diagram of the eighth α helices of the PsaA and PsaB proteins from *Synechocystis* 6803 and the regions containing the proposed ligands to the [4Fe-4S] center F_X. Site-directed residue changes described in the text are shown next to arrowheads. F_X is depicted as a cube. Regions predicted to form α helices are bounded by rectangles. Dashed lines represent the possible leucine zipper interaction. Solid lines represent the cysteinyl-iron bond coordinating the [4Fe-4S] center.

cyanobacteria are excellent for the study of photosynthesis. By selecting under light-activated heterotrophic growth (LAHG) conditions (Anderson and McIntosh, 1991), we have isolated targeted mutants in which either the *psaA* or the *psaB* gene (which encode the PSI reaction center proteins in *Synechocystis* 6803) have been inactivated (Smart *et al.,* 1991, see Chapter 3; Smart and McIntosh, 1992, see Chapter 4). The ability to recover mutants lacking the PSI reaction center indicates that the *psaA* and *psaB* genes are viable candidates for site-directed mutagenesis. Inactivation mutations have also been introduced individually into the *psaD*, *psaE*, and *psaF* genes, encoding peripheral PSI polypeptides in *Synechocystis* 6803 (Chitnis *et al.,* 1989a; Chitnis *et al.,* 1989b; Chitnis *et al.,* 1991), but those mutants exhibited essentially wild-type characteristics. Systems for the genetic inactivation of PSI genes have also been developed using another cyanobacterium, *Anabaena variabilis* sp. ATCC 29413 (Toelge *et al.,* 1991; Mannan *et al.,* 1991), and the green alga *Chlamydomonas reinhardtii* (Bingham *et al.,* 1991; Takahashi *et al.,* 1991).

We will describe the first site-directed amino acid changes in a PSI reaction center protein incorporated *in vivo.* Site-directed mutations targeted to a proposed ligand of F_X and to a leucine zipper motif were incorporated into the *psaB* gene, and mutants were selected under LAHG conditions. Analysis of these mutants provides new insight into the biogenesis and structure of the PSI reaction center.

MATERIALS AND METHODS

Materials

Chemicals and antibiotics were of high purity and were purchased from Sigma Chemical Co. (St. Louis,MO) or Research Organics (Cleveland, OH). Restriction and other enzymes were purchased from New England Biolabs (Beverly, MA), bacto-agar from Difco (Detroit,MI), and nitrocellulose from Schleicher and Schuell (Keene,NH). Radioisotopes ([α-^{32}P]dATP and [α^{35}-S]dATP) were purchased from Amersham (Arlington Heights,IL).

Strains and growth conditions

Experiments were performed using a glucose-tolerant strain of *Synechocystis* sp. PCC 6803, which was acclimated for growth on solid medium in the dark (Williams, 1988; Anderson and McIntosh, 1991). Except for tests for autotrophy, cells were grown at 30°C under light-activated heterotrophic growth (LAHG) conditions, as previously described (Anderson and McIntosh, 1991), except that a 10-min pulse of light from fluorescent bulbs (rather than 5-min) was used to ensure full illumination of all cultures in the growth chamber. Cultures were grown in BG-11 medium (Allen, 1968) supplemented with 5 mM glucose and 5 mM *N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES). 1.5% w/v purified bacto-agar (Anderson and McIntosh, 1991) and 3 g/liter sodium thiosulfate were added to solid medium. Antibiotics were added in the following concentrations: kanamycin (Km), 5 mg/liter; spectinomycin

(Sp), 20 mg/liter. Transformations were performed essentially as described (Williams, 1988), except in the case of strain ΔB-RCPT, which was carefully maintained in dim light throughout the procedure, since it is very light-sensitive. Tests for autotrophic growth were performed using both liquid and solid medium without supplemental glucose in a chamber providing continuous light (≈20 $\mu mol \cdot m^{-2} \cdot s^{-1}$). To ensure that cells would grow in continuous light, *i.e.* that they were not light-sensitive, cultures were also grown in the light in medium with glucose. For those strains that exhibited light-sensitivity, screens were used to reduce the light intensity to approximately 3 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Large cultures were grown in 15 liters of medium in carboys, bubbled with air; were harvested using a continuous flow rotor (DuPont Sorvall, Wilmington, DE); and were frozen at -70°C in BG-11 with 15% v/v glycerol.

DNA manipulations

Nucleic acids were manipulated using standard methodology (Sambrook *et al.,* 1989), unless otherwise stated. *E. coli* strains XL1-Blue (Stratagene, La Jolla, CA) and TG1 (Amersham, Arlington Heights, IL) were used. Site-directed mutagenesis was performed using an oligonucleotide-directed *in vitro* mutagenesis kit as directed by the manufacturer (Amersham, Arlington Heights, IL). DNA sequencing was performed using Sequenase version 2 polymerase as directed by the manufacturer (United States Biochemical, Cleveland, OH). The polymerase chain reaction (PCR) was performed using a Perkin-Elmer Cetus thermal cycler and AmpliTaq polymerase as directed by the manufacturer

(Perkin-Elmer, Norwalk, CT). Asymmetric PCR amplification was performed according to McCabe (1990). Amplification products were extracted once with chloroform to remove mineral oil. Unincorporated nucleotides were removed by precipitating and centrifuging the amplification products with an equal volume (100 μl) of 4 M ammonium acetate and 2 volumes of ethanol. The method for large-scale preparation of cyanobacterial DNA has been described (Smart, Bowlby, Anderson, Sithole, and McIntosh, submitted; see Chapter 5). Small-scale preparations of DNA were performed on cells either scraped from solid medium or harvested from 50-ml liquid cultures as described (Ohad and Hirschberg, 1992).

Membrane isolation and analysis

Thylakoid membranes were isolated using a modification of a previously described procedure (Smart *et al.,* 1991, see Chapter 3). Material was maintained on ice or at 4°C throughout the procedure. Previously frozen cells from a 15-liter culture were centrifuged in a Sorvall GSA rotor (8000 R.P.M., 8 min), and the pellet resuspended in approximately 100 ml of break buffer (50 mM Tris, pH 8.3; 0.8 M sucrose) to which were added: RNase A (20 μg/ml), DNase I (20 μg/ml), phenylmethylsulfonylfluoride [PMSF] (10 μg/ml), and N-tosyl-L-phenylalanine-chloromethyl ketone [TPCK] (10 μg/ml). The cells were broken in a Bead Beater (Biospec Products, Bartelsville, OK) at 0°C with approximately 200 ml of glass beads using 10 cycles of 30 sec on, followed by 8 min off. The cell extract solution was decanted, and the beads were repeatedly

washed with break buffer, which was decanted again, until the total volume of the extract and buffer was 800 ml. Cell debris and remaining glass beads were pelleted by centrifugation at 6000 R.P.M. for 8 min in a Sorvall GSA rotor. The supernatant fluid was decanted into clean tubes, then 1 M calcium chloride and 50% w/v PEG-8000 were added to a final concentration of 25 mM and 5%, respectively. The resulting solution was centrifuged for 25 min at 12,000 R.P.M. in a Sorvall GSA rotor. The supernatant fluid, containing most of the phycobilisomes, was discarded, and the membrane-containing pellets were resuspended in glycerol freeze buffer (50 mM Tris, pH 8.3; 20 mM calcium chloride; 25% v/v glycerol). This solution was centrifuged at 18,000 R.P.M. for 30 min in a Sorvall SS34 rotor, and the supernatant fluid was discarded. The final pellets were resuspended in glycerol freeze buffer, pooled, and frozen at -70°C.

SDS-PAGE and immunoblotting were performed as previously described (Smart *et al.*, 1991, see Chapter 3). To resolve the PsaA/B proteins, 10% SDS-PAGE gels were used; 17% gels were used to resolve PsaC and PsaD. Protein assays were performed using the method of Lowry *et al.* (1951). Equal amounts of protein (150 μg) was loaded in each lane. Antibodies raised in rabbits to the PsaA/B proteins from *Synechococcus* were previously described (Henry *et al.*, 1990). Antibodies were raised in rabbits to PsaC or PsaD protein purified from strains of *E. coli* expressing, respectively, the *psaC* gene from *Synechococcus* sp. PCC 7002 or the *psaD* gene from *Nostoc* sp. PCC 8009 (Li *et al.*, 1991). PS I activity was measured in a Rank Brothers oxygen electrode

(Cambridge, England) using a modification of methods described by Izawa (1980). Light (from projector lamps) was passed through a copper sulfate solution and filtered with red cellophane, providing saturating illumination. Membranes equivalent to 2.5 μg chlorophyll (chl) were assayed in 1 ml of buffer (50 mM Hepes, pH 7.5; 10 mM NaCl; 5 mM $MgCl_2$) at 25°C containing 1 mM KCN, 20 μM N_1-(dichlorophenyl)-N_3-dimethylurea (DCMU), 10 μg/ml superoxide dismutase (Boehringer Mannheim, Indianapolis, IN), 0.5 mM sodium ascorbate, 0.1 mM 2,6-dichlorophenolindophenol (DCIP), and 1.0 mM methyl viologen. Chl was extracted with methanol and quantified using published extinction coefficients (Lichtenthaler, 1987). A PSI complex was isolated from thylakoid membranes using 1% v/v Triton X-100 and differential centrifugation, followed by sucrose density centrifugation in 0.1% Triton X-100, as described (Golbeck and Cornelius, 1986).

ESR spectroscopy

Room temperature ESR spectroscopy was performed on a Bruker ER200D spectrometer essentially as previously described (Barry and Babcock, 1987). Membranes were pelleted and resuspended in 50 mM Tris, pH 8.3; 10 mM NaCl; 5 mM $MgCl_2$; 7.5% w/v PEG-3400 (Aldrich, Milwaukee, WI). Illumination was provided by a high-intensity microscope lamp. Spectra were mathematically manipulated using an IBM PS/2 computer and SpectraCalc software (Galactic Industries Corp.). Low temperature ESR spectroscopy was performed using a Bruker ECS-106 X-band spectrometer equipped with an

Oxford liquid helium cryostat and temperature controller, essentially as previously described (Zhao *et al.,* 1992). Illumination was provided by a 150-W xenon lamp.

RESULTS

Recipient strain

To allow for rapid segregation of mutations in the *psaB* gene, a recipient strain of *Synechocystis* 6803 was engineered with the region of the chromosome targeted for mutagenesis deleted. A plasmid (pLS3531K) was constructed that incorporates a fragment encoding kanamycin resistance (Km^R) from the plasmid pUC4K (Vieira and Messing, 1982; Taylor and Rose, 1988), flanked by a fragment encoding the 3'-half of *psaA* and the 5'-half of *psaB* from the plasmid pLS35 and a fragment containing the *psaB* 3'-flanking region from the plasmid pLS31 (Fig 2A). Wild-type *Synechocystis* 6803 was transformed with pLS3531K, and Km^R colonies were selected under LAHG conditions. After five rounds of streaking from single colonies, DNA was isolated from one of the Km^R colonies and was subjected to Southern analysis using a fragment of the *psaB* gene as a probe. The pattern of hybridization confirmed the segregation of the deletion mutation (data not shown). This strain (ΔB-RCPT) exhibited the turquoise-blue color characteristic of cells lacking PSI due to genetic inactivation of either *psaA* or *psaB* (Smart *et al.,* 1991, see Chapter 3; Smart and McIntosh, 1992, see Chapter 4).

Site-directed mutagenesis

Plasmids for site-directed mutagenesis of the *psaB* gene were constructed *in vitro* and manipulated in *E. coli*. Oligonucleotides were designed to effect the desired change in the coding sequence, while also destroying or adding a restriction site. The resulting change in digestion pattern serves as an effective and simple means of screening for the desired mutation. Site-directed mutations were made using the Amersham oligonucleotide-directed *in vitro* mutagenesis system and single-stranded DNA generated from the plasmid pLS15, which contains a 1.2 kilobase pair (kbp) *Hin*d III fragment encoding the 3'-half of *psaB* (Smart and McIntosh, 1991, see Chapter 2). Incorporation of the desired mutations was verified by restriction mapping and by DNA sequencing. A 940-bp *Afl* II/*Eco*R I fragment from each mutated pLS15 plasmid was subcloned into the *Afl* II/*Eco*R I sites of the plasmid pLS35. The Ω fragment, which encodes resistance to spectinomycin and streptomycin (Sm^R/Sp^R), from the plasmid pHP45Ω (Prentki and Krisch, 1984) was cloned into an *Afl* II site in pLS31, forming the plasmid pLS31Ω. This *Afl* II site lies 100 bp downstream from the termination codon of *psaB* in the *Synechocystis* 6803 chromosome. An *Eco*R I fragment from pLS31Ω was cloned into the *Eco*R I site of pLS35 and the mutated pLS35 plasmids. This final construct, plasmid pLS3531Ω (Figure 6.2B), contains DNA that spans the deletion in the strain ΔB-RCPT and includes a selectable marker, the Ω cassette, downstream from the *psaB* gene. The mutated pLS3531Ω plasmids were double-checked by restriction mapping and by DNA sequencing to verify the presence of the

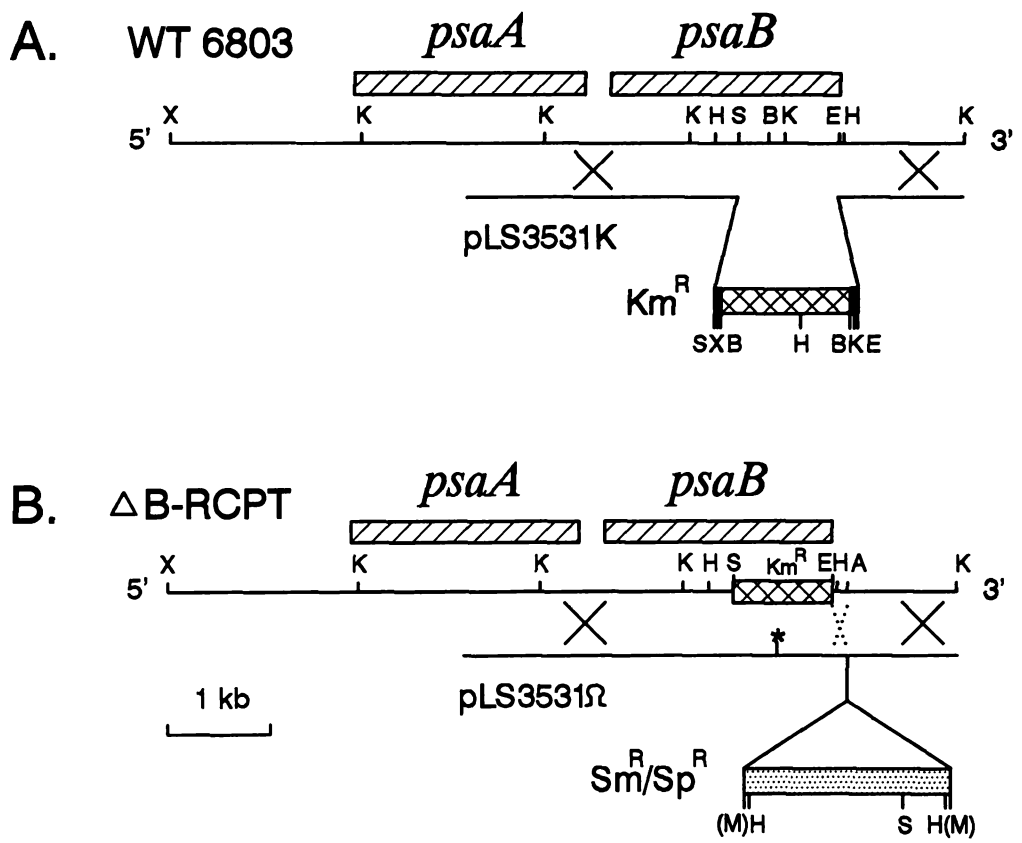

Figure 6.2. Partial restriction maps of *psaA-psaB* and of plasmids for mutagenesis. **A.** Map of the insert region from the plasmid pLS3531K and of the wild-type *Synechocystis* 6803 *psaA-psaB* operon. **B.** Map of the insert region from the plasmid pLS3531Ω and of the *psaA-psaB* region of the chromosome in strain ΔB-RCPT. Hatched boxes represent the coding regions of *psaA* and *psaB*. Crossed-hatched boxes represent the Km^R cassette from the plasmid pUC4K. The stippled box represents the Ω cassette from the plasmid pHP45Ω. The solid regions of the cross-hatched rectangle in A represent portions of the pUC119 polylinker. Crosses indicate possible regions of homologous recombination. The asterisk indicates the location of a site-directed mutation. Restriction sites are: X, *Xba* I; K, *Kpn* I; H, *Hin*d III; S, *Sph* I; B, *Bam*H I; E, *Eco*R I; M, *Sma* I. Sites in parentheses were destroyed in cloning.

desired mutations and the proper sequence. Plasmid pLS3531Ω and its mutated variants were then used to transform the strain ΔB-RCPT, and Sp^R colonies were selected under LAHG conditions.

The presence of the desired mutations in Sp^R colonies was verified by three different methods, each having particular advantages. To confirm the incorporated change in restriction pattern quickly and from small batches of cells, small-scale preparations of DNA were used as templates for conventional PCR amplification of the mutated region of the *psaB* gene. The resulting PCR products were digested with the informative restriction enzymes, resolved by gel electrophoresis, and visualized by ethidium bromide staining (data not shown). To confirm the overall integrity of the entire *psaB* region of the chromosome and to detect any wild-type copies of the *psaB* gene, genomic DNA was digested with the informative restriction enzyme and other enzymes recognizing sites in this region and subjected to Southern analysis, using a fragment of the *psaB* gene as a probe (data not shown). Finally, to verify the proper integration of *psaB* sequence back into the region deleted in the strain ΔB-RCPT, this region of DNA was amplified from CsCl-gradient-purified genomic DNA by asymmetric PCR (McCabe, 1990), and the primarily single-stranded PCR products were sequenced directly using internal oligonucleotide primers (data not shown). This technique would also have the capability to detect a mixture of wild-type and mutant sequence in genomic DNA. In addition to initial verification of the mutants, DNA was isolated from samples taken from the large cultures to be used for phenotypic characterization to confirm that

reversion had not occurred. In all of the cultures tested, no mixture of wild-type and mutant sequence or reversion of mutants to wild type was ever detected.

Characterization

Mutations were made in the *psaB* gene as depicted in Figure 6.1, changing the Cys at position 565 to Ser (C565S), His (C565H), and Asp (C565D); changing the Leu at position 522 to Val (L522V) and Pro (L522P); changing the Leu at position 536 to Met (L536M); and introducing mutations at both positions 522 and 536 (L522V/L536M). To serve as a control, the strain ΔB-RCPT was transformed with the wild-type plasmid pLS3531Ω (Figure 6.2B), yielding the strain WTΩ. After genetic verification of each mutant strain, cells were tested for their ability to grow autotrophically in the light (≈20 $\mu mol \cdot m^{-2} \cdot s^{-1}$) without glucose. The three Cys mutants and L522P, which all exhibited light-sensitivity, failed to grow autotrophically (Table 6.1). The three conservative Leu mutants, L522V, L536M, and L522V/L536M, all grew autotrophically at near wild-type rates (Table 6.1). PSI electron transport activity was measured in an oxygen electrode using isolated membranes. The rate of O_2 uptake was measured in the presence of: $DCIPH_2$, an electron donor to PSI; methyl viologen, an electron acceptor from PSI; and inhibitors of PSII activity (DCMU) and cytochrome oxidase activity (KCN) (Izawa, 1980). The rates of O_2 uptake by isolated membranes from L522V and L522V/L536M were slightly lower than from the WTΩ control, while the rate of O_2 uptake by

Table 6.1. Characterization of *psaB* mutants.

Strain	Autotroph	Rates of O_2 uptake*	O_2 uptake per cell†	Chl per cell‡	Chl/prot in membrane§
WTΩ	Yes	2353±287	1624	0.69±0.10	10.71±2.47
C565S	No	765±185	459	0.60±0.21	12.98±4.01
C565H	No	289±30	127	0.44±0.01	8.78±1.62
C565D	No	309±60	164	0.53±0.02	8.41±2.62
L522V	Yes	1991±102	1234	0.62±0.08	11.20±1.88
L536M	Yes	1648±289	1467	0.89±0.23	16.36±2.84
L522V/L536M	Yes	1905±266	1372	0.72±0.09	12.29±1.97
L522P	No	274±34	142	0.52±0.02	8.42±1.63

*(μmol O_2·(mg chl)$^{-1}$·h^{-1}) Average of at least five assays from at least two preparations.

†(μmol O_2·(OD_{730})$^{-1}$·liter^{-1}·h^{-1}) Product O_2 uptake rate and chl per cell.

‡(mg chl·(OD_{730})$^{-1}$·liter^{-1}) Average of at least four assays from at least two different cultures.

§(μg chl·(mg prot)$^{-1}$) Average of at least two assays from at least two preparations.

membranes from L536M was slightly lower still, when expressed on the basis of chlorophyll (Table 6.1). The rates of O_2 uptake by isolated membranes from C565H, C565D, and L522P were all approximately equal and were very low relative to WTΩ (Table 6.1). The rates from these three strains were comparable to rates measured using isolated membranes from strain BDK8, in which the *psaB* gene has been inactivated (Smart and McIntosh, 1992, see Chapter 4). However, the rates from strain C565S were over two-fold higher than those from the other two Cys mutants, but were still significantly lower than WTΩ, when normalized to chlorophyll (Table 6.1). When expressed on a per cell basis, the rates of O_2 uptake by isolated membranes from L522V, L536M, and L522V/L536M were comparable to WTΩ, and the rates from C565H, C565D, and L522P were all equally very low (Table 6.1). When expressed on a per cell basis, the rate from C565S was approximately one-third that of WTΩ and was three-fold higher than those of the other Cys mutants (Table 6.1).

The reduced rates of O_2 uptake measured using membranes from C565S, C565H, C565D, and L522P could have been the result of the lack of stable assembly of PSI or the assembly of PSI with reduced activity. One way to determine the accumulation of the chlorophyll-binding reaction center proteins in cyanobacteria is to quantify the accumulation of chlorophyll, both in whole cells and relative to the amount of protein in isolated membranes. The strains C565H, C565D, and L522P all had reduced amounts of chlorophyll, both in the whole cell measurements and in isolated membranes (Table 6.1).

Amounts of chlorophyll were slightly reduced in the strains C565S and L522V, when measured in whole cells, while the strain L522V/L536M had near wild-type levels of chlorophyll per cell (Table 6.1). Chlorophyll determinations of cells and membranes from strain L536M were consistently slightly higher than wild-type (Table 6.1). The relative amounts of chlorophyll in the membranes generally followed the same trends seen in the whole cell measurements (Table 6.1). The mutants C565S, L522V, L536M, and L522V/L536M were green in color, while C565H, C565D, and L522P exhibited a turquoise-blue color (not shown). Variability in the chlorophyll determinations may have been introduced by differing accumulation of PSII or by the presence of free chlorophyll.

In order to assay the accumulation of PSI proteins more precisely, immunoblotting of membrane proteins was performed using antibodies recognizing the proteins PsaA/B, PsaC, or PsaD (Figure 6.3). The cross-reaction of all three antibodies to protein from strains L522V, L536M, and L522V/L536M appeared to be approximately equal to that of wild type. All three antibodies recognized reduced levels of protein in membranes from C565S relative to wild type. However, greatly reduced amounts of the PsaA/B proteins were detected in membranes from C565H and C565D, and PsaA/B were undetectable in membranes from L522P. The PsaC and PsaD proteins were not detected by antibodies in membranes from C565H, C565D, or L522P. However, the PsaD antibody did recognize a protein of approximately 11 kDa with equal intensity in all the samples. Very faint cross-reaction to a protein

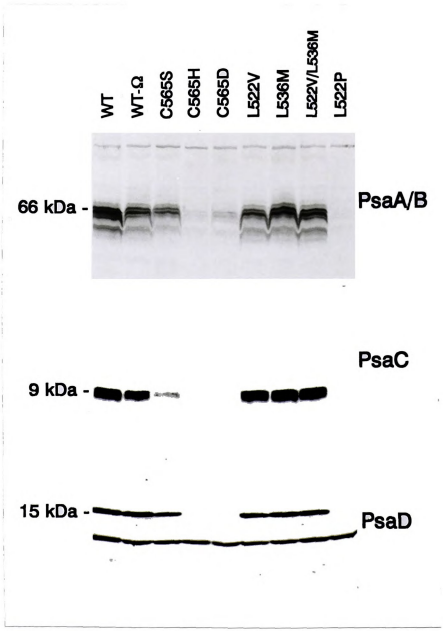

Figure 6.3. Immunoblots of membrane proteins probed with antibodies recognizing PsaA/B, PsaC, or PsaD. Equal amounts of protein (150 μg) were loaded in each lane. M_r was estimated by comparison to migration of prestained molecular weight standards (Diversified Biotech, Newton Centre, MA) and is expressed in kiloDaltons (kDa).

with approximately the same M_r has been seen with different antiserum recognizing PsaD (Smart *et al.,* 1991, see Chapter 3), suggesting the presence of another protein with an epitope in common with PsaD.

Room temperature ESR spectroscopy was utilized to detect signal I, from P_{700}^+ (Figure 6.4). Signal I has a typical linewidth of 0.8 - 1.0 milliTesla (mT) centered at g=2.0025 (Norris *et al.,* 1971) and can overlap signal II from tyrosine Y_D^+ in PSII ($\Delta H_{pp} \approx 2.0$ mT, g=2.0046) (Barry and Babcock, 1987). Therefore, a spectrum of membranes from the strain BDK8, an inactivation mutant of *psaB* (Smart and McIntosh, 1992, see Chapter 4), was scaled and subtracted from the normalized spectra of each strain, in order to remove signal II from the spectra. The spectra from L522V, L536M, and L522V/L536M are essentially the same as that of WTΩ, with a feature typical of signal I and of approximately equal intensity. The spectrum of membranes from strain L522P lacks any prominent features, once signal II has been subtracted. The spectra of membranes from the three cysteine mutants include a feature of similar intensity, with a lineshape, linewidth, and approximate g value expected for signal I.

ESR spectroscopy was also performed at cryogenic temperatures to detect signals generated by reduction of the [4Fe-4S] centers F_A and F_B bound by the PsaC protein. F_A is characterized by g values of 2.05, 1.94, and 1.86, while F_B has g values of 2.07, 1.92, and 1.89 (Malkin and Bearden, 1978). When both centers are reduced, their high-field and low-field signals merge, producing a spectrum with g values of 2.05, 1.94, 1.92, and 1.89 (Malkin and

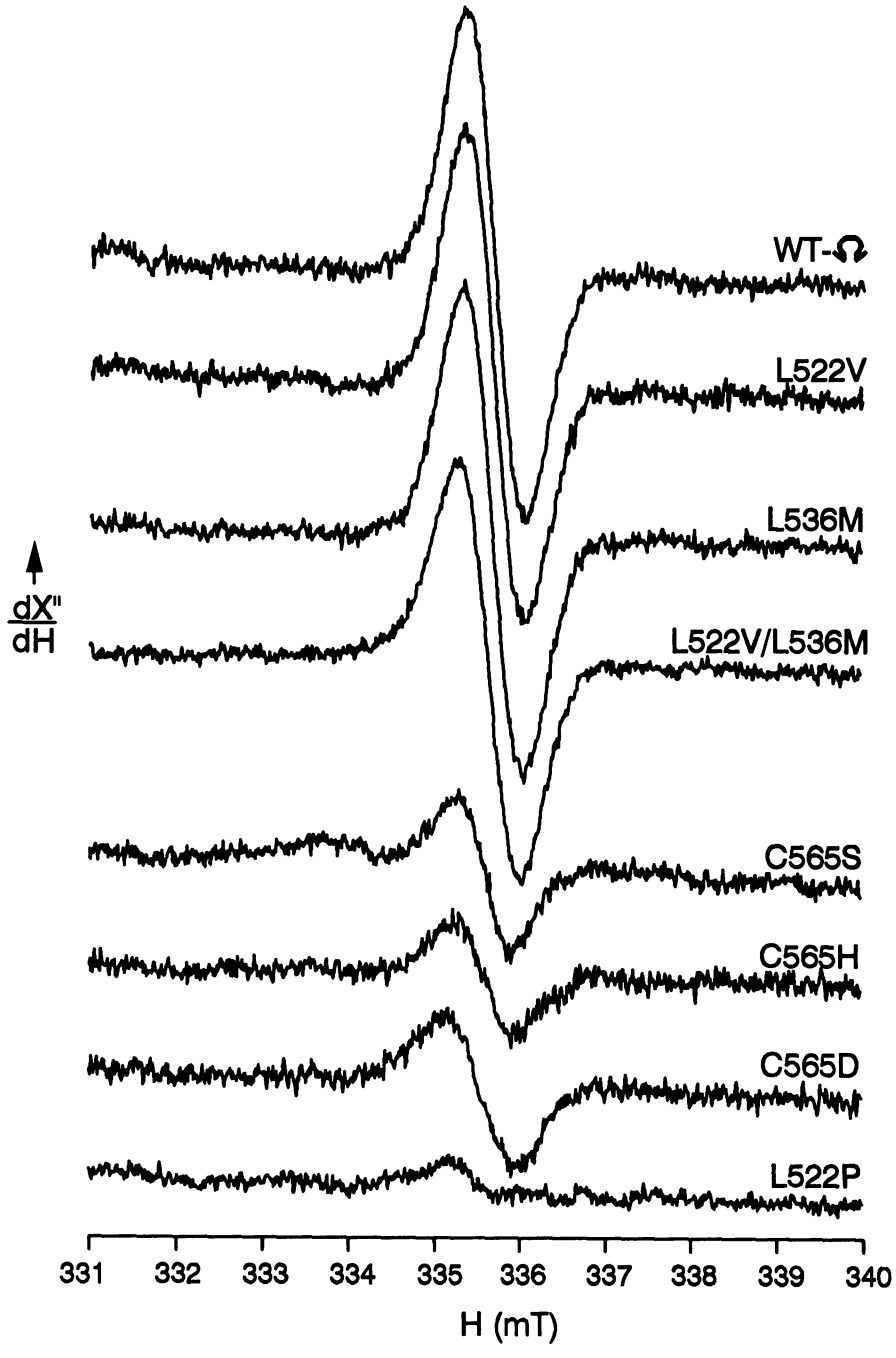

Figure 6.4. Room temperature ESR spectra taken during illumination of thylakoid membranes. Spectra were normalized to approximately equal protein and equal spectrometer gain, then a scaled spectrum of BDK8 membranes (Smart and McIntosh, 1992, see Chapter 4) was subtracted. The magnetic field (H) is expressed in milliTesla (mT) Spectrometer conditions were: power, 20 mW; field modulation, 0.4 mT; time constant, 200 ms; sweep time, 200 s.

Bearden, 1978). The low temperature spectra of membranes from strains L522V, L536M, and L522V/L536M were essentially wild-type, while F_A/F_B signals were not present in spectra of membranes from the strains C565H, C565D, and L522P (see Appendix). Signals from photoreduced F_A/F_B were present in spectra of membranes from C565S (see Appendix) and in spectra of PSI complex isolated from C565S membranes (Figure 6.5). In both cases, the F_A and F_B resonances were present at reduced intensity relative to those in the spectrum of PSI complexes isolated from wild-type membranes. The *g* values of the features in the C565S spectrum are essentially the same as wild-type, as is the ratio of the intensities of resonances from F_A and F_B (see Appendix).

DISCUSSION

Site-directed mutagenesis of PSI

We have described the first *in vivo* site-directed amino acid changes in a PSI protein. This mutagenesis was rapidly accomplished using the unicellular cyanobacterium *Synechocystis* 6803, which has an active homologous recombination system (Williams, 1988) and may be grown heterotrophically (Anderson and McIntosh, 1991). By first generating a deletion recipient strain, site-directed mutations could be integrated into the chromosome efficiently, avoiding the problem of segregation of a mixture of wild-type and mutant sequences. These mutants appear to be quite stable, as we have not yet detected reversion of any mutations. *Synechocystis* 6803 also has the advantage

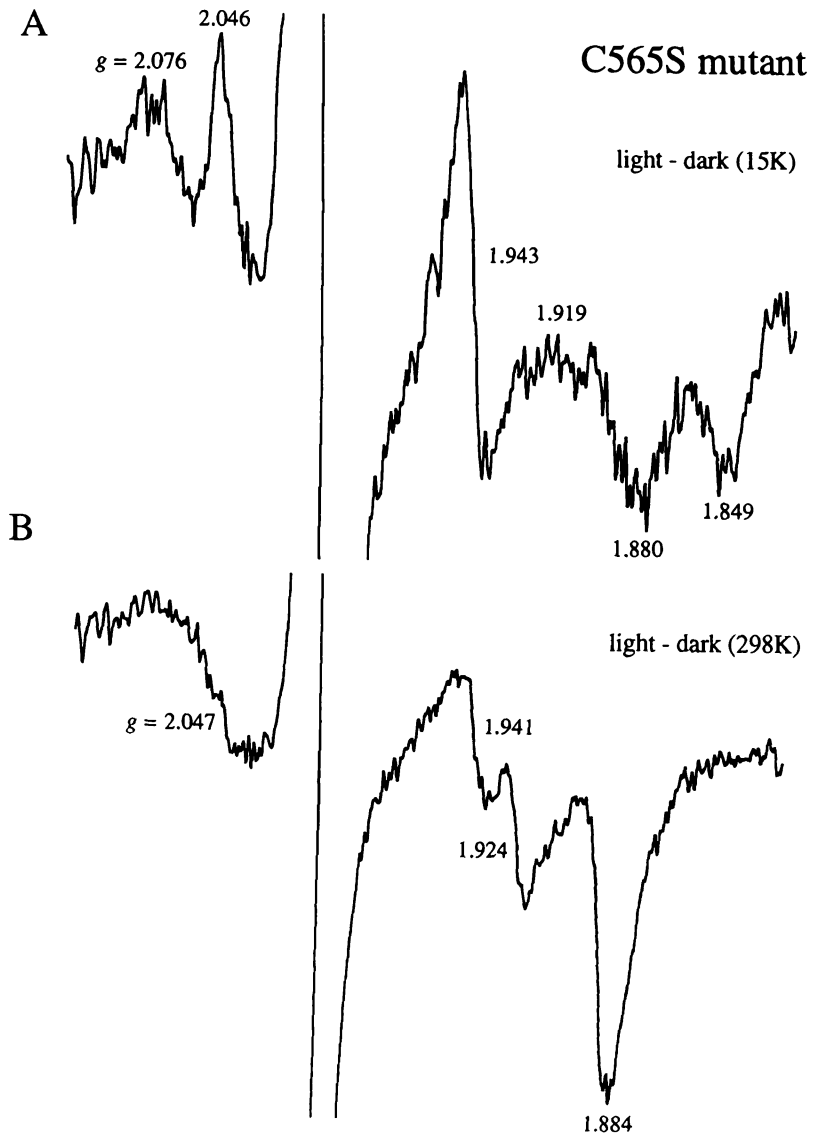

Figure 6.5. Low temperature ESR spectra of PSI complex isolated from the C565S mutant. Spectra were taken at 15 K under illumination of (**A**) complex frozen in the dark and (**B**) complex frozen under illumination. Numbers above and below the spectra indicate the *g*-values of the principal resonances. The spectrum in A was multiplied by a factor of 2 relative to that in B.

of well characterized procedures for the purification of both PSI and PSII (Rögner *et al.,* 1990; Noren *et al.,* 1991).

Changes of the cysteine residue at position 565 of PsaB have profound effects on the stable assembly of PSI. The mutants C565S, C565H, and C565D all have greatly reduced accumulation of the PsaA, PsaB, PsaC, and PsaD polypeptides, as determined by chlorophyll assays and immunoblotting. Also, PSI activity is greatly reduced or absent in the three cysteine mutants as assayed by O_2 uptake assays and ESR spectroscopy. Assaying for the ability of these strains to grow autotrophically is complicated by their sensitivity to continuous light above ≈ 3 $\mu mol \cdot m^{-2} \cdot s^{-1}$. These strains will grow in dim light (< 3 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with glucose, but the low light intensity in combination with reduced amounts of assembled PSI may not allow autotrophic growth, even if photosynthetic electron transport were functional in the mutants. The mutant C565S does accumulate significantly more PSI than the other two cysteine mutants, reflected in higher chlorophyll accumulation, higher O_2 uptake rates and stronger signals on immunoblots. Electron transfer to the terminal electron acceptors in PSI, F_A and F_B, was detected in complexes from C565S, but the amount of F_A and F_B photoreduced is less than in wild-type complex on a chlorophyll basis. The reduced intensity of signal I may be explained by rapid rereduction of P_{700}^+ due to impaired forward electron flow.

Synechocystis 6803 appears to have an active mechanism for digesting or degrading PSI subunits that cannot form an active PSI complex. This is evident in the L522P mutant in which none of the assayed PSI polypeptides

accumulated. This phenomenon may be sensitive to improper protein folding or perhaps to redox status. In contrast, expression of mutant *psaC* in *E. coli* has allowed purification of mutant PsaC protein, which may then be reconstituted with the PSI core protein *in vitro* (Zhao *et al.,* 1990; Zhao *et al.,* 1992). Using this system, substitution of an aspartate for a cysteine ligand to either F_A or F_B causes an inactive [3Fe-4S] center to form, rather than a [4Fe-4S] center (Zhao *et al.,* 1992). However, the *in vitro* reconstitution system requires great excess of added PsaC, PsaD, iron, and sulfur and may lack important components and interactions integral to the natural system, and thus may not truly reflect the *in vivo* situation.

The three residue substitutions at position 565 represent the non-cysteinyl residues found to coordinate iron-sulfur centers in naturally occurring or mutant iron-sulfur proteins. Histidine residues, in addition to cysteines, serve as ligands to the [2Fe-2S] centers in the Reiske iron-sulfur protein from *Thermus thermophilus* (Cline *et al.,* 1985), spinach (Britt *et al.,* 1991), and *Saccharomyces cerevisiae* (Graham and Trumpower, 1991). An aspartate, as well as cysteines, serves as a ligand to one of the iron-sulfur centers in ferredoxins from *Desulfovibrio africanus* (George *et al.,* 1989) and *Pyrococcus furiosus* (Conover *et al.,* 1990). This center exists in the [3Fe-4S] state until activated, when it reacts with Fe(II) ion to form a [4Fe-4S] center (George *et al.,* 1989; Conover *et al.,* 1990). Site-directed substitutions of serines for cysteines that ligate center 1 in *E. coli* fumarate reductase altered the spectroscopic and electrochemical properties of the protein, but still effectively

coordinated the [2Fe-2S] centers (Werth *et al.,* 1990). However, substitutions of serine for cysteine in center 2 of the *E. coli* fumarate reductase or in the *E. coli* dimethyl sulfoxide reductase prevented formation of [4Fe-4S] centers. Interestingly, in the latter protein, a [3Fe-4S] center does form (Manodori *et al.,* 1992; Rothery and Weiner, 1991). In addition, the [4Fe-4S] center in activated pig heart aconitase is coordinated by three cysteine residues and either water or hydroxyl from the solvent (Robbins and Stout, 1989). It is important to note that all of these examples represent iron-sulfur centers with intra-subunit ligands. The F_X center is a rare example of inter-subunit coordination of an iron-sulfur center.

The conservative mutations introduced into the leucine zipper motif of PsaB had no detectable effect on PSI accumulation or activity. All the assayed characteristics of the mutants L522V, L536M, and L522V/L536M were essentially wild-type, including chlorophyll accumulation, O_2 uptake activity, ESR signal I intensity, protein accumulation as determined by immunoblotting, autotrophic growth rate, and photoreduction of F_A/F_B as assayed by ESR spectroscopy. We did not specifically assay PSI stability under conditions that might disrupt the leucine zipper, such as elevated temperature, although PSI complex from the L522V, L536M and L522V/L536M mutants behaved like wild-type complex through detergent solubilization and PSI purification (not shown).

The residue substitutions incorporated into the leucine zipper motif have been effective in perturbing leucine zippers in other proteins. In the case of

C/EBP, single and double valine for leucine substitutions disrupted dimerization, as measured by cross-linking (Landschulz *et al.*, 1989). Likewise, single and double valine substitutions in the protein Jun reduced dimerization with Fos, as measured by co-immunoprecipitation with antibodies to either Fos or Jun (Gentz *et al.*, 1989). However, heptad repeats of leucines are also found in ion channels and do not appear to act primarily in subunit dimerization (McCormack *et al.*, 1989; McCormack *et al.*, 1991). Single valine for leucine substitutions in the *Drosophila* Shaker K^+ channel protein altered properties of the voltage-dependent channel opening, implying a role of the repeated leucines in pore formation or channel gating (McCormack *et al.*, 1991). The leucine zipper proteins that have been mutated so far have been, for the most part, soluble proteins. The leucine zipper motif in the PSI reaction center is a rare example of a membrane intrinsic leucine zipper. One may not be able to extrapolate from the results obtained by previous study of soluble leucine zippers when analyzing the effects of mutations in a membrane-bound leucine zipper. The specific interactions between leucines may be less important in a membrane environment, or fewer leucines may be required to accomplish dimerization. Also, it is unclear whether a similar coiled-coil structure would form in a membrane-bound leucine zipper.

In contrast to the introduction of conservative substitutions, the introduction of a proline at position 522 of PsaB had a deleterious effect on the biogenesis of PSI. This mutant has virtually undetectable levels of PsaA, PsaB, PsaC, and PsaD; no ESR signal I; low accumulation of chlorophyll; low rates of

O_2 uptake; and no detectable reduced F_A/F_B ESR signals. In all aspects measured, this strain has the phenotype of BDK8, an inactivation mutant of *psaB* (Smart and McIntosh, 1992, see Chapter 4). The leucine to proline residue substitution probably disrupts the secondary structure of the eighth α-helix of PsaB, preventing stable assembly of the PSI reaction center and accumulation of PSI proteins. However, the codon introduced in this substitution is used only 6% of the time compared with the other codons for proline in a survey of 29 *Synechocystis* 6803 genes (not shown). Low abundance of the tRNA for this codon may also contribute to the lack of accumulation of PsaB in strain L522P.

Conclusions

Our data indicate that the cysteine at position 565 of PsaB serves as a ligand to the [4Fe-4S] center F_X and supports the proposal that F_X bridges the PSI reaction center proteins (Golbeck and Cornelius, 1986; Golbeck, 1992). Not only does F_X act as an electron transfer component, but this [4Fe-4S] center also plays a major structural role in reaction center dimerization and PSI complex stability. Our data do not definitively address the role of the leucine zipper, but suggest one of the following conclusions: 1) the mutations introduced (other than L522P) were too conservative to disrupt the leucine zipper interaction, 2) the leucine zipper was disrupted, but plays a minor role in PSI biogenesis, perhaps acting functionally in an as yet unmeasured capacity, or 3) the leucine zipper does not form in the PSI reaction center of *Synechocystis*

6803. Further mutagenesis, already in progress, will be needed to address definitively the leucine zipper hypothesis.

The successful completion of this series of site-directed mutations in the *psaB* gene invites further genetic manipulation of the PSI reaction center, hopefully leading to a full understanding of the structure-function relationships important for the assembly and activity of this intricate and vital protein complex.

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CHAPTER 7

DISCUSSION AND CONCLUDING REMARKS

This project has accomplished many of the goals established at its inception: the isolation and characterization of the PSI reaction center genes from *Synechocystis* 6803, the inactivation of those genes, and site-directed mutagenesis of a PSI reaction center gene to study structure-function relationships. Yet, as with nearly all scientific endeavors, many unresolved questions have arisen in the process of addressing the initial problems. It is also important to consider the questions that warrant future experimentation. The following is a brief discussion of some of those questions left unresolved and of possible experiments that may address some of the interesting problems that remain. Many of the proposals in this section are based on speculation, rather than definitive evidence.

Coordinate regulation of thylakoid membrane complexes

Growth of *Synechocystis* 6803 under LAHG conditions causes significant changes in the balance of proteins in the thylakoid membrane. There is a three- to four-fold decrease in the accumulation of PSI, while the accumulation

of PSII remains approximately the same as in mixotrophically grown cells. Patterns of mRNA accumulation suggest some level of post-transcriptional regulation of PSI and PSII biogenesis, since *psaA-psaB* mRNA accumulation remains approximately the same, while *psbA* mRNA accumulation drops significantly. The accumulation of PSI seems to be the variable component, rather than the levels of PSII, when *Synechocystis* 6803 is acclimating to changes in its environment. Variation in PSI accumulation has also been detected when *Synechocystis* 6803 is grown in different light quality, as well (Aizawa *et al.*, 1992). The changes in PSI accumulation are probably caused by differential protein stability or complex stability under conditions which favor more or less PSI. Thus, under certain redox conditions, PSI may be more or less stable, and its accumulation is adjusted to allow for the most efficient use of available light energy for photosynthetic electron flow. An additional consideration in cyanobacteria is the coordinate regulation of the accumulation of the cytochrome b_6/f complex, which is shared between photosynthesis and respiration. The relative accumulation of these proteins under photoautotrophic versus LAHG conditions has not been studied extensively.

Light sensitivity of PSI mutants

Mutants of *Synechocystis* 6803 that lack PSI, including ADK9, BDK8, and L522P, are sensitive to continuous light >3 $\mu mol \cdot m^{-2} \cdot s^{-1}$. This suggests that PSI and its associated pigments are important for photoprotection. Initially the light-sensitivity was thought to be caused by photoinhibitory damage

resulting from full reduction of PSII and the quinone pool. However, mutants that lack both PSI and PSII are also sensitive to continuous light >3 $\mu mol \cdot m^{-2} \cdot s^{-1}$, eliminating any role of PSII and P_{680} in causing photoinhibitory damage. Thus, two possibilities come to mind: 1) there is some light-induced component creating a toxic element in the cells, or 2) continuous illumination >3 $\mu mol \cdot m^{-2} \cdot s^{-1}$ prevents respiratory electron flow and ATP synthesis. A possible toxic component may be a pigment, such as chlorophyll or carotenoid, which is no longer associated with a photosynthetic complex and can create toxic radicals or molecular oxygen when illuminated. The second possibility cannot be distinguished from the first at this time, because the only mutants that lack PSI activity also lack all of the chlorophyll associated with PSI. If one had a mutant, which accumulated wild-type levels of PSI that was inactive in cyclic photosynthesis and ATP production, one could test the light-sensitivity of the respiratory pathway *in vivo* directly in cells with a normal complement of chlorophyll.

Comparison of PSI to other photosynthetic reaction centers

The photosynthetic reaction centers from the purple bacteria *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* have been crystallized, and their three-dimensional structures have been determined (Allen *et al.,* 1987; Deisenhofer *et al.,* 1985). This is important for the study of oxygenic photosynthesis, because there is significant structural and functional homology between the bacterial reaction center L and M subunits and the PSII

subunits D1 and D2, suggesting an evolutionary relationship (Michel and Deisenhofer, 1988). Comparison of PSII to the bacterial reaction center crystal structure has guided mutational analysis of PSII structure and function (Debus *et al.,* 1988; Vermaas *et al.,* 1988). Recent evidence suggests there are photosynthetic reaction centers that are evolutionarily related to PSI in the green sulfur bacterium *Chlorobium* and in the anaerobe *Heliobacillus mobilis* (Trost *et al.,* 1992; Feiler *et al.,* 1992). The reaction centers in these organisms appear to be homodimers that contain iron-sulfur centers that act in electron transport (Nitschke *et al.,* 1990a; Nitschke *et al.,* 1990b). The genes encoding the reaction center proteins in *Chlorobium limicola* f.sp. *thiosulfatophilum* have been isolated and characterized (Büttner *et al.,* 1992). There are three genes in an operon, two of which encode reaction center proteins of 730 and 232 amino acids. The larger protein shares some homology with PsaA and PsaB, especially in the region containing the cysteines that bind the iron-sulfur center F_X. The smaller protein has limited homology with PsaC, but contains similar cysteine motifs that would bind the iron-sulfur centers F_A and F_B. The gene encoding the reaction center from *Heliobacillus mobilis* has also been isolated and partially characterized (Wilson *et al.,* 1991). This protein, with a mass of approximately 47 kDa, is much smaller than PsaA or PsaB, but does have a similar pair of cysteines that bind F_X. The entire twelve-residue motif containing the cysteine ligands to F_X is not conserved in the reaction center proteins from the two organisms. Of note, the aspartates adjacent to the cysteines are altered, as is an arginine between the two cysteines. Interestingly,

these proteins do not contain heptad repeats of leucines, proposed to form a leucine zipper in PSI. Although it is interesting to make broad comparisons between these reaction center proteins and PSI, only the *Chlorobium* protein will be useful on a limited scale in judging which amino acids may be important for PSI structure and function. The *Heliobacillus* protein appears to be too divergent. Also, the state of purification and crystallization of PSI is far ahead of that of either *Chlorobium* or *Heliobacillus.*

Comparisons have been made between the bacterial reaction center, PSII, and PSI using computer algorithms (Otsuka *et al.,* 1992; Margulies, 1991). The authors conclude that there is significant similarity between regions of the PSII and PSI reaction center proteins. However, the regions of similarity discovered in the two studies do not correspond. One of the papers proposes that there are 13 membrane-spanning helices in PsaA and PsaB, rather than the widely accepted number of 11. Preliminary interpretation of the crystal structure of *Synechococcus* PSI suggests that there may only be eight membrane-spanning helices (J. Golbeck, personal communication). This lack of consistency and the comparison to PSII, for which no three-dimensional structure exists, lead to the conclusion that these computer comparisons do not provide a strong basis for predicting which amino acids are important for PSI structure and function. Although, they are interesting when considering the evolutionary relationships between different types of photosynthetic reaction centers.

Potential future experiments

The most interesting site-directed mutant generated in this project is C565S. It still accumulates PSI that is capable of low temperature electron transfer to F_A and F_B, but reduced F_X has eluded detection to this point. Also, preliminary time-resolved optical spectroscopy suggests that F_A and F_B are not photo-reduced at room temperature (see Appendix). Since the reaction center does assemble, although at reduced levels, there probably is an iron-sulfur center forming. However, that cluster may be a [3Fe-4S] center, which would not transfer electrons at room temperature and may be EPR silent. The reduction of F_A and F_B seen at low temperature may represent the low temperature bypass of A_1 and F_X that has been detected before (Setif *et al.,* 1987). Therefore, further experiments, including EPR spectroscopy and time-resolved optical spectroscopy are needed to define the nature of F_X in this mutant and to investigate the role of F_X in room temperature and low temperature electron transport. In addition, studies involving electron-nuclear double resonance (ENDOR) spectroscopy, Mössbauer spectroscopy, or electron spin echo envelope modulation (ESEEM) spectroscopy could further define the nature of the iron-sulfur cluster and its ligands.

Mutations that would alter the environment and the redox potential of F_X, without affecting PSI stability, would be ideal for the study of electron flow through the reaction center. Amino acid changes that might accomplish this include changing the aspartate residues adjacent to the cysteine ligands of F_X, perhaps to asparagines, serines, or cysteines. Another residue that may be

involved in determining the environment around F_X is the arginine between the two cysteines, which might be changed to lysine or leucine. Replacement of a cysteine ligand of the [4Fe-4S] center in ferredoxin I from *Azotobacter vinelandii* for alanine by site-directed mutagenesis resulted in protein rearrangement, allowing a different cysteine residue to act as a ligand to the [4Fe-4S] cluster (Martin *et al.,* 1990). A similar approach could be attempted with the ligands to F_X, replacing a cysteine with alanine or glycine, while simultaneously changing a nearby amino acid to cysteine. This might force a minor protein rearrangement in order for F_X to bind, which could alter the environment and character of F_X. Amino acids in this region may also be involved in the association with PsaC, so the substitutions described above may alter electron donation to F_A and F_B as well.

Further mutagenesis is required to address definitively the role of the repeats of leucines in PSI. Amino acid substitutions that are less likely to participate in the leucine zipper interaction include: glycine, alanine, or phenylalanine. Also more double leucine substitutions, including isoleucine or alanine residues may be effective. A mutation, which is currently being attempted, is the deletion of the leucine residue at position 522 of PsaB, which would disrupt the spacing of the leucines in the array. Also, the addition of a codon in the region predicted to form the α helix may perturb the leucine zipper without significantly altering the secondary structure of that region. A recipient strain of *Synechocystis* 6803 and plasmids for the site-directed mutagenesis of the *psaA* gene have been engineered and may be used for the

introduction of amino acid substitutions into PsaA (unpublished data).

The investigation of structure-function relationships in the PSI reaction center could be expanded beyond the study of F_X and the leucine zipper, but with less likelihood of immediate success. Residues that act as ligands to P_{700}, A_0, and A_1 have been proposed (Margulies, 1991; Otsuka *et al.,* 1992) and may be discovered by site-directed mutagenesis. It is in the study of these electron transfer components that comparison of PSI to *Chlorobium* or PSII may provide insight for successful mutagenesis.

The understanding of oxygenic photosynthesis is vital for our continued prosperity in a changing environment. This study has advanced the knowledge of photosynthesis in a cyanobacterium, but our ultimate goal must be the full understanding of photosynthesis in plants, which are vital for our survival. The recently developed system for transformation of tobacco chloroplasts opens the door for analysis of structure-function relationships in the plant PSI reaction center using site-directed mutagenesis. Although the cyanobacteria serve well for rapid mutagenesis and analysis, study of these organisms must be considered preliminary to the study of plants. Ultimately, we may be able to alter the photosynthetic reaction centers in a positive way, possibly increasing efficiency, stability, or high-light tolerance. Perhaps the plant photosynthetic apparatus could be engineered for the production of H_2 to be used as fuel. Hopefully, this study has provided some useful information that may be used some day to accomplish these goals.

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APPENDIX

APPENDIX

ESR and optical spectroscopy of wild type and site-directed mutants

In order to observe reduction of the [4Fe-4S] centers F_A and F_B in either isolated membranes or PSI complex, ESR spectroscopy was performed at 15 K using a Bruker ECS-106 spectrometer equipped with an Oxford cryostat and temperature controller (see Chapter 6). The low temperature ESR spectrum of wild-type PSI complex that was frozen in the dark includes resonances originating from reduced F_A (g=2.046, 1.944, and 1.852) or reduced F_B (g=2.067, 1.927, and 1.881) (Figure A.1, top panel). When the wild-type PSI complex was frozen under illumination, the high-field and low-field resonances from F_A and F_B merge, yielding a spectrum with features at g=2.048, 1.940, 1.921, and 1.885 (Figure A.1, bottom panel). The low temperature ESR spectra of isolated membranes from the mutants L522V, L536M, and L522V/L536M frozen under illumination have essentially wild-type character (Figure A.2, panels A, C, E, and G). The spectrum of membranes from C565S has resonances with reduced intensity, relative to those of wild type, while reduced F_A or F_B signals were not present in the spectra of membranes from C565H, C565D, or L522P (Figure A.2, panels B, D, F, and H). The low

temperature spectra of membranes isolated from C565S include weak F_A and F_B signals, when illuminated at 15 K (Figure A.3, panel A). When C565S membranes are frozen under illumination, the high- and low-field resonances merge, but the intensity of the mid-field feature from F_B appears to be very weak (Figure A.3, panel B). The spectrum of membranes from C565S that were chemically reduced using dithionite includes F_A and F_B features that are more intense than those observed upon photoreduction, suggesting that electron transfer to F_A and F_B may occur at reduced efficiency (Figure A.3, panel C). Finally, 85% of the transient, flash-induced absorption change at 820 nm, reflective of reduction of P_{700}, of PSI complex purified from membranes of C565S decays with a halftime of 4 μs, while the remaining 15% decayed with a longer halftime, on the order of ms (Figure A.4, panel A). This indicates impaired forward electron transfer through F_X, resulting in a rapid back-reaction (perhaps from A_1^-) and rereduction of P_{700}. With wild-type PSI complex, the flash-induced absorption change decays with a halftime on the order of tens of ms, reflecting the back-reaction of F_A/F_B^- with P_{700} (Figure A.4, panel B).

Acknowledgements

ESR and optical spectroscopy was performed by Patrick Warren in the laboratory of Dr. John Golbeck, Department of Biochemistry, University of Nebraska.

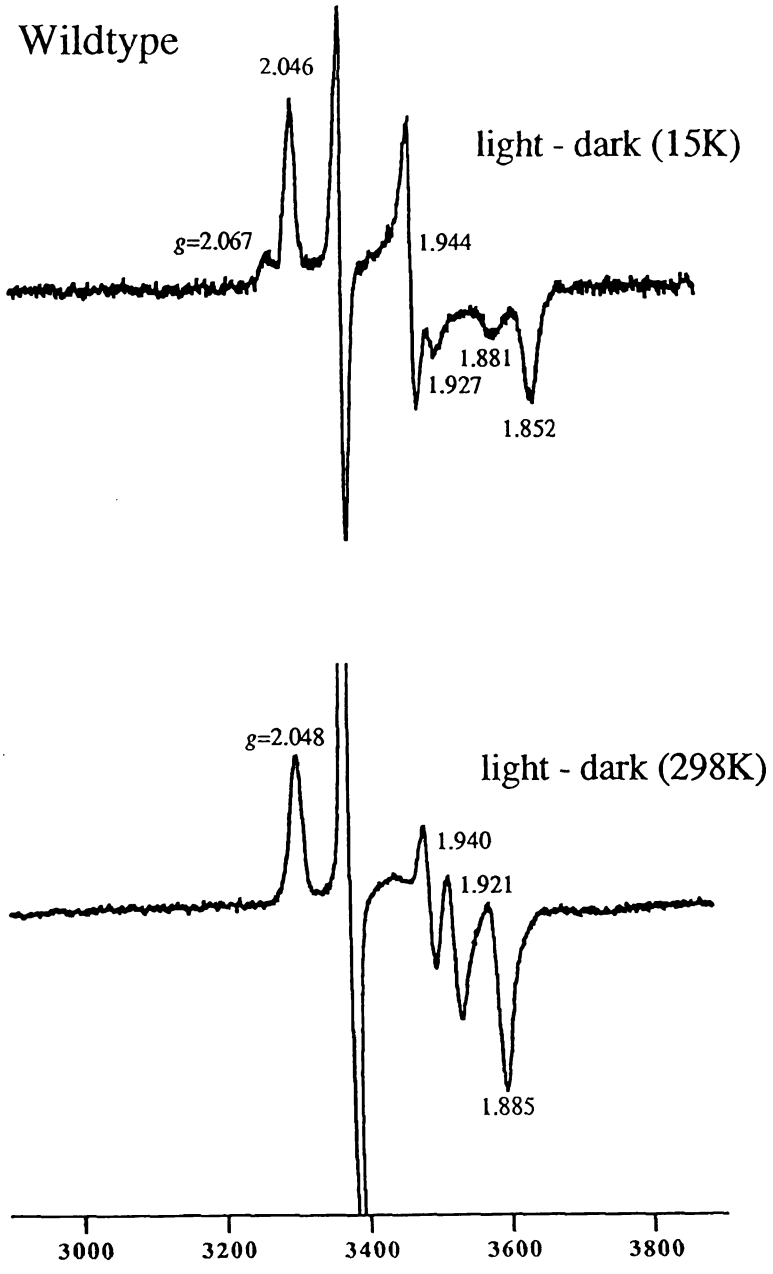

Figure A.1. Low temperature ESR spectra of wild-type PSI complex. The top spectrum was collected during illumination after the sample was frozen in the dark. The bottom spectrum was collected under illumination after the sample was frozen under illumination. The *g* values are indicated above and below features in the spectra. The magnetic field (horizontal axis) is indicated in Gauss.

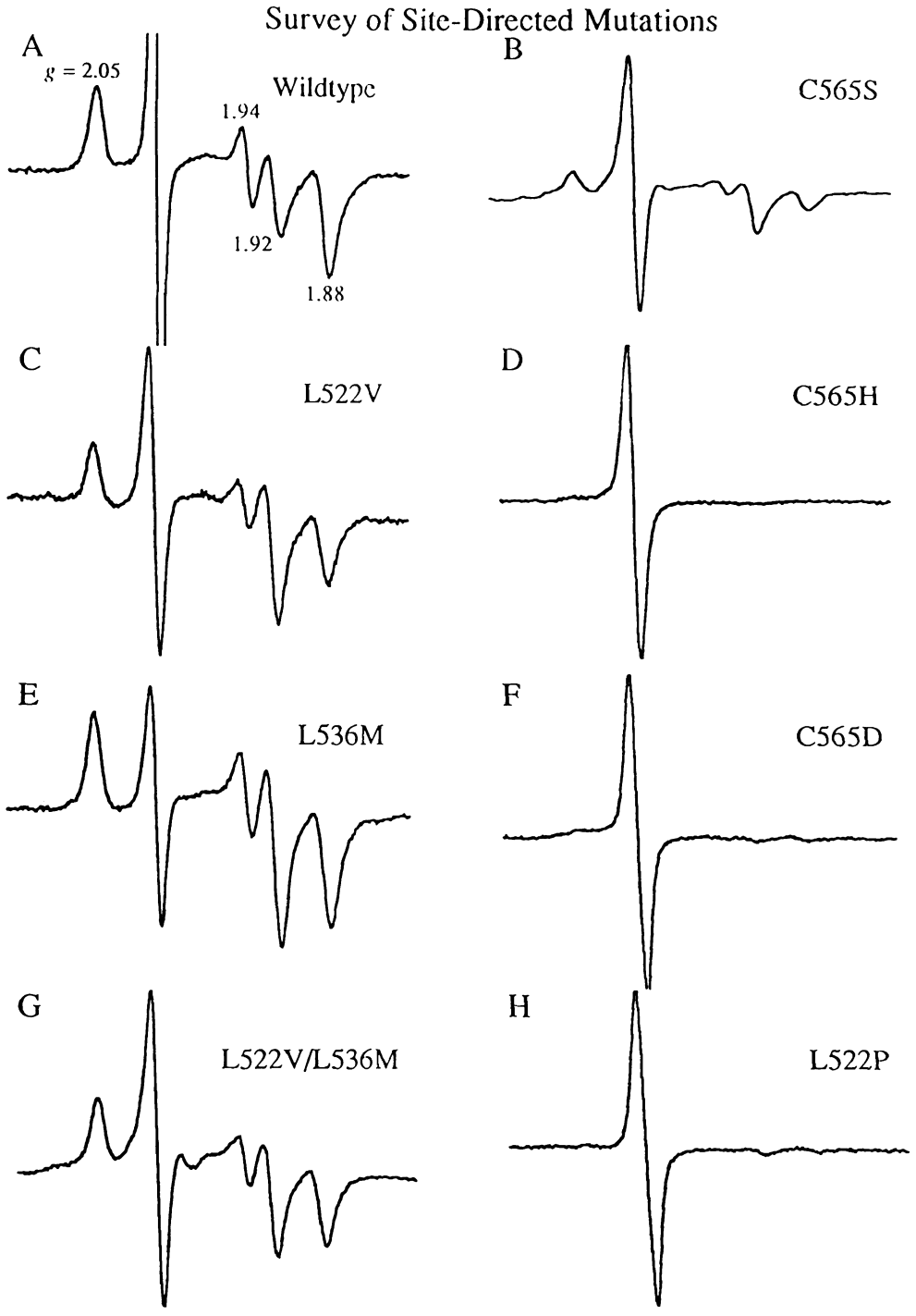

Figure A.2. Low temperature ESR spectra of membranes isolated from wild-type and mutant cells. The spectra were collected under illumination after the sample was frozen under illumination.

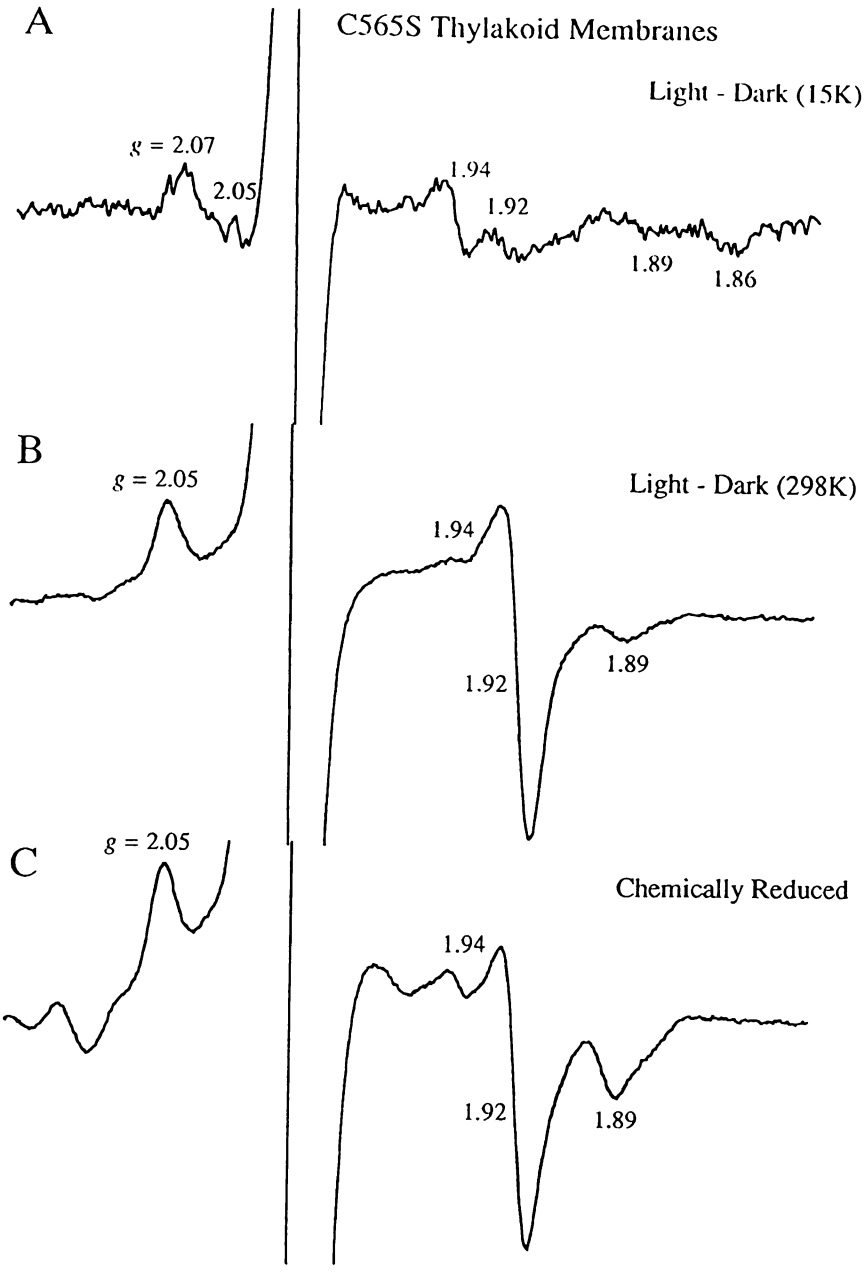

Figure A.3. Low temperature ESR spectra of membranes isolated from C565S. The spectrum in A was collected during illumination after the sample was frozen in the dark. The spectrum in B was collected under illumination after the sample was frozen under illumination. The spectrum in C was collected after chemical reduction with dithionite in the presence of 0.1 M glycine, pH 10. The *g* values are indicated above and below the spectra.

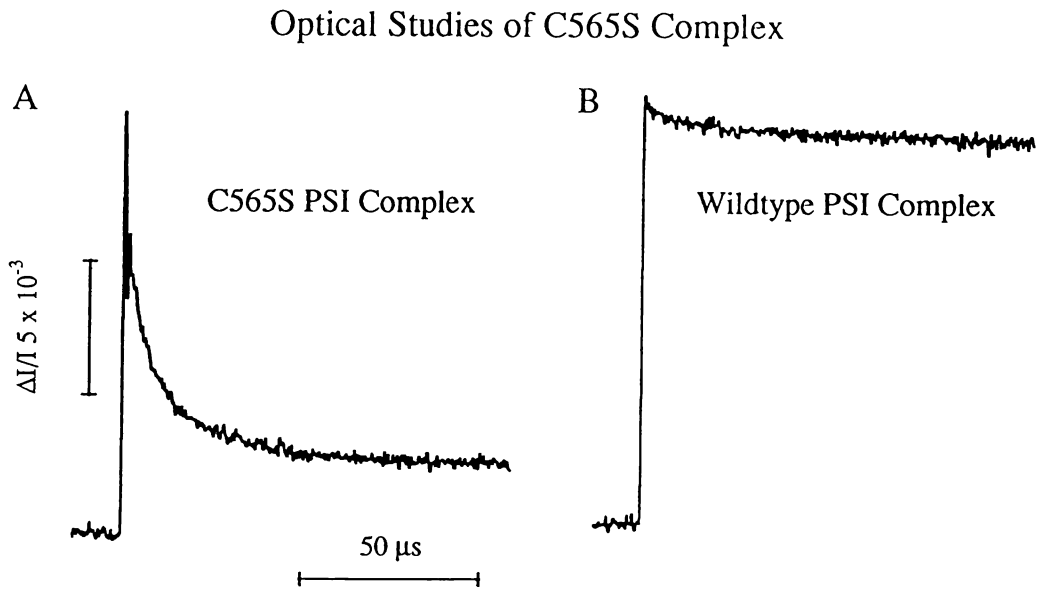

Figure A.4. Optical spectroscopy of PSI complex isolated from C565S and wild type. The transient, flash-induced absorption change was measured at 820 nm using a double beam spectrometer. The measuring beam was supplied by a Schwartz Electro-Optics titanium-sapphire laser pumped by a 5 W CW argon-ion laser (Spectra-Physics) and was split, one beam passing through the sample to a detector, the other passing directly to a matching detector. The signals were amplified and subtracted using a DC-coupled differential comparator (Tectronix model 11A33, 150 MHz) and were digitized with a Tectronix DSA 601 oscilloscope. Samples were excited using a 2.3 MW nitrogen laser (PTI) operating at 1 Hz and contained 0.1 mM DCIP and 5mM ascorbate in 0.1 M Tris, pH 8.3.