High Sensitivity Proteomics Assisted Discovery of a Novel Operon Involved in the Assembly of Photosystem II, a Membrane Protein Complex.*§

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Photosystem II (PSII) is a large membrane protein complex that performs the water oxidation reactions of photosynthesis in cyanobacteria, algae, and plants. The unusual redox reactions in PSII often lead to damage, degradation, and reassembly of this molecular machine. To identify novel assembly factors, high sensitivity proteomic analysis of PSII purified from the cyanobacterium *Synechocystis* sp. PCC 6803 was performed. This analysis identified six PSII-associated proteins that are encoded by an operon containing nine genes, slr0144 to slr0152. This operon encodes proteins that are not essential components of the PSII holocomplex but accumulate to high levels in pre-complexes lacking any of the luminal proteins PsbP, PsbQ, or PsbV. The operon contains genes with putative binding domains for chlorophylls and bilins, suggesting these proteins may function as a reservoir for cofactors needed during the PSII lifecycle. Genetic deletion of this operon shows that removal of these protein products does not alter photoautotrophic growth or PSII fluorescence properties. However, the deletion does result in decreased PSII-mediated oxygen evolution and an altered distribution of the S states of the catalytic manganese cluster. These data demonstrate that the proteins encoded by the genes in this operon are necessary for optimal function of PSII and function as accessory proteins during assembly of the PSII complex. Thus, we have named the proteins of the slr0144-slr0152 operon Pap (Photosystem II assembly proteins).

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†The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1 and Table 1.

‡The data from the proteomics analysis of isolated PSII complexes from the HT3, ΔpsbP HT3, ΔpsbQ HT3, and ΔpsbV HT3 strains have been deposited with NCBI GEO and can be found under GEO accession number GSE9577.

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‡2 The abbreviations used are: PSII, Photosystem II; Pap, Photosystem II assembly protein; ORF, open reading frame; AMT tag, accurate mass and time tag; LC, liquid chromatography; Chl, chlorophyll; pD1, D1 precursor protein; TES, 2-[2-(hydroxyethyl)(dimethylamino)ethanesulfonic acid; FTICR, Fourier transform ion cyclotron resonance; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MS, mass spectrometry; WT, wild type.

Photosystem II (PSII)§ is the multicomponent enzyme complex in cyanobacteria, algae, and plants that catalyzes the light-driven oxidation of water to molecular oxygen. The active complex is a dimer consisting of 2 identical monomers with more than 20 proteins, identified through genetic, biochemical, and structural studies. In addition to its protein components, PSII also has a large number of cofactors including chlorophylls, plastobilins, plastocyanines, manganese atoms, calcium, chloride, non-heme iron, and heme groups (1, 2). Removal of these subunits or cofactors can slow or even completely halt water oxidation.

The assembly of this crucial complex is an intricate process. The steps of protein assembly into the complex are ordered and well regulated (3). D2, cytochrome P680, and PsbT bind to form a receptor complex into which the D1 precursor protein (pD1) is inserted (4, 5). The CP47 protein then joins the pre-complex followed by the low molecular weight proteins PsbH, PsbM, and PsbTc. The C-terminal extension of pD1 is then processed by the luminal protease CtpA into the mature D1 protein (4). Next, the CP43 and PsbK proteins associate. At this point the soluble luminal extrinsic proteins (PsbO, PsbP, PsbQ, PsbU, and PsbV) can also bind to the complex. These luminal proteins are not essential for photosynthesis but are located in close proximity to the site of water oxidation, enhance oxygen evolution, and have roles in protecting the catalytic manganese cluster from damage (6, 7). The presence of these proteins can be viewed as an indicator of the functional state of the complex, as PsbQ is solely associated with dimerized complexes that are fully assembled and highly active (8). Finally, the monomeric complex dimerizes. Fig. 1 shows a simplified schematic of PSII assembly in cyanobacteria.

The steps involved in the association of cofactors with the functional complex are less defined. The mechanism of integration of some of these cofactors has been well documented, as is the case for the manganese cluster. It is known that after the incorporation of the CP43 protein, all of the ligands for the MnCaCl cluster are present within the complex, and pre-
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FIGURE 1. Schematic of PSII assembly. The pD1 protein is inserted into the pre-complex containing D2. CtpA processes pD1 and CP47 joins the complex. The last core protein CP43 is then assembled. The luminal proteins PsbP, PsbQ, PsbO, PsbV, and PsbU then bind, forming the functional PSII complex.

summarily integration of this catalytic center occurs. However, the integration of other cofactors, including the insertion of chlorophyll into the chlorophyll-containing proteins D1, D2, CP47, and CP43, remains undefined.

In addition to the initial assembly of the subunit proteins and cofactors into this large membrane complex, PSII is frequently damaged in the course of its natural function (9). The D1 protein becomes irreversibly damaged, and the luminal proteins and the catalytic manganese cluster must be dissociated so that the damaged D1 protein can be removed and a new copy inserted into the complex (10). Because many of the PSII cofactors would be highly detrimental in large quantities if free in the cell, it is likely there are chaperone proteins which sequester these cofactors before assembly and during the repair and degradation of the PSII complex. As an example, small CAB (chlorophyll A/B binding)-like proteins have recently been proposed to sequester chlorophylls from damaged PSII complexes until they are recycled into new complexes (11). It is likely that intermediaries may also hold other cofactors of the complex during complex repair. Thus, in addition to the complexity of its composition, PSII also has an intricate lifecycle of repair and degradation. Therefore, besides the stoichiometric components of the holocomplex necessary for enzyme activity, other accessory proteins must associate with PSII throughout its lifecycle to repair the protein components and recycle cofactors.

Although high resolution crystal structures are available for cyanobacterial PSII (1, 2), not all of the biochemically identified proteins and cofactors have been visualized in the current structures. Indeed the most comprehensive of these structures only displays 20 proteins and 77 cofactors per monomer (2). Previous analysis in *Synechocystis* sp. PCC 6803 utilizing the strain HT3, which contains a hexahistidine tag on the core membrane protein CP47 (12), has identified 31 polypeptides associated with active PSII complexes using denaturing electrophoretic separation followed by matrix-assisted laser desorption ionization mass spectrometry (MS) and N-terminal amino acid sequencing (13). This analysis revealed that there were non-characterized PSII-associated proteins that represent non-stoichiometric proteins that transiently associate with the complex as well as stoichiometric complex members not part of the crystallized complex.

In this study we undertook a global proteomics analysis of isolated PSII complexes, comparing protein profiles of HT3 to those of Δ*psbV* HT3, Δ*psbP* HT3, and Δ*psbQ* HT3 in *Synechocystis* 6803. These mutants contain PSII complexes that have been arrested at the currently understood end point of assembly (see Fig. 1). The sensitivity of these techniques allowed for identification of not only the stoichiometric components of active PSII complexes but also for the identification of proteins transiently associated with PSII throughout its lifecycle, such as assembly, repair, or degradation partners. From the results, we identified an operon of unknown function that contains binding domains for photosynthetic cofactors. This operon is systematically conserved among cyanobacteria. Deletion of the operon shows that although it is not required for photoautotrophy, it does stabilize photosynthetic capacity, indicating a function in PSII-mediated activity. We have named the products of this operon Photosystem II assembly proteins (Pap).

**EXPERIMENTAL PROCEDURES**

**Growth Conditions**—All chemicals used for media and other experiments are from Sigma unless otherwise noted. *Synechocystis* cultures were grown at 30 °C under 20 μmol of photons m⁻² s⁻¹ white fluorescent light in TES-buffered BG11 medium (14). Stock cultures were maintained on solid medium (BG11 supplemented with 1.5% (w/v) agar) and used to inoculate liquid cultures for each experiment. When needed for mutants, growth medium was supplemented with 10 μg/ml spectinomycin, 2 μg/ml gentamycin, and 10 μg/ml chloramphenicol. To create a putative mass tag library, cultures were grown in complete BG11 as well as in media deplete of nitrogen, phosphorous, sulfur, or iron; media containing sodium chloride, 3-(3,4-dichlorophenyl)-1,1-dimethyleurea, or carbon dioxide or cultures were subjected to heat or cold shock.

**PSII Preparation**—PSII was isolated from HT3, Δ*psbV* HT3, Δ*psbQ* HT3, and Δ*psbP* HT3 strains as described previously (13). HT3 indicates the His₆-tagged CP47 used to affinity purify the complex (12). The final eluate from a nickel-nitrilotriacetic acid-agarose (Qiagen, Inc., Valencia, CA) column was suspended in 50 mM MES-NaOH, pH 6.0, 10 mM CaCl₂, 25% glycerol with 0.04% dodecyl maltoside.

**Spectrophotometric Assays**—Cell growth was monitored by measuring light scattering at 730 nm on a μQuant microplate spectrophotometer (Bio-Tek Instruments, Inc., Toronto, Canada). Chlorophyll (Chl) concentrations were determined by methanol extraction and absorbance at 652 and 665 nm in a DW2000 spectrophotometer (SLM-Aminco, Urbana, IL) (15).

**Accurate Mass and Time (AMT) Tag Peptide Identification**—To create the AMT tag library, cellular samples were digested with sequencing grade-modified trypsin (Promega, Madison, WI) using a ratio of 1:100 (w/w) protease to protein sample for 4 h at 37 °C. Digests were desalted using Supelco Supercleat SCX tubes (St. Louis, MO), and the pH of each digestion was adjusted to 3.5 using formic acid. The SCX resin was conditioned with acetonitrile followed by 1 M sodium formate. The column was washed 25% acetonitrile in 500 mM ammonium acetate, pH 8.5, and re-equilibrated with 5% aceto-
nitrile in 10 mM ammonium formate, pH 3.5. Peptide mixtures were loaded onto the resin and washed with 5% acetonitrile in 10 mM ammonium formate, pH 3.5. Peptides were eluted with 25% acetonitrile in 500 mM ammonium acetate, pH 8.5, followed by 100% acetonitrile. Eluted peptides were concentrated via SpeedVac (ThermoSavant, San Jose, CA) to protein concentrations of 1.0 mg/ml, as determined by BCA assay (Pierce).

Peptides were putatively identified using a capillary liquid chromatography (LC) system of a pair of model 100 ml 100DM syringe pumps (Teledyne-Lsco, Lincoln, NE), a series D controller (Teledyne-Lsco), and an in-house manufactured mixer, capillary column selector, and sample loop. Separations were achieved using a 5000 p.s.i. reversed-phase in-house-packed capillary (150-μm inner diameter, 360-μm outer diameter, 60 cm long; Polymicro Technologies, Phoenix, AZ) by using an exponential gradient of 2 mobile-phase solvents consisting of 0.2% acetic acid and 0.05% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in 90% acetonitrile. Flow through the capillary high performance liquid chromatography column was ∼1.8 μl/min when equilibrated to 100% mobile-phase 0.2% acetic acid and 0.05% trifluoroacetic acid.

For each sample, 10 μg was infused into a LCQ conventional ion trap MS (ThermoFinnigan, San Jose, CA) operating in a data-dependent MS/MS mode over a 400–2000 m/z range. For each cycle, the 3 most abundant ions from MC analysis were selected for MS/MS analysis by using a collision energy setting of 45%. Dynamic exclusion was used to discriminate against previously identified ions. The collision-induced dissociation spectra from the conventional ion trap mass spectrophotometer were analyzed using SEQUEST (16) and the genome sequence of Synechocystis 6803 (17). Putative mass tag identifications were made based on a SEQUEST cross correlation (Xcorr) score ≥2.0, regardless of charge or mass.

Using the same LC conditions, 5 μg of sample analyzed in the ion trap was then analyzed in duplicate or triplicate by FTICR-MS. The FTICR mass spectrometers use ESI interfaced with an electrodynamic ion funnel assembly coupled to a radio frequency quadrupole for collisional ion focusing and highly efficient ion accumulation and transport to a cylindrical FTICR for cell analysis (18).

The resultant FTICR data were processed using the PRISM Data Analysis system, software tools developed in-house. First the MS data were de-isotoped, giving the monoisotopic mass, charge, and intensity of the major peaks in each mass spectrum. Then the data were examined in a two-dimensional fashion to find groups of mass spectral peaks that were observed in sequential spectra. Each group, known as a unique mass class, has a median mass, central normalized elution time, and abundance estimate, computed by summing the intensities of the MS peaks that compromise the unique mass class. The identity of each unique mass class was determined by comparing the mass and normalized elution time of each unique mass class with the mass and normalized elution time of the 4423 putative mass tags in the Synechocystis 6803 AMT tag data base (generated using the peptides observed from 23 LC/MS/MS). Search tolerances were ±6 ppm for the mass and ±5% of the total run time for the elution time.

PSII Proteomics—PSII preparations of ∼1 mg of protein were sent to Pacific Northwest National Laboratory for protein identification. The PSII samples were denatured by the addition of equal volumes of 7 M urea, 2 M thiourea, and 1% CHAPS in 50 mM ammonium bicarbonate, pH 7.8, and then reduced with dithiothreitol to a final concentration of 5 mM. CaCl₂ was added to a final concentration of 1 mM. Samples were digested and analyzed utilizing the LC/MS process as described for AMT peptide identification above.

The peak matching process gives a list of peptide matches and observed abundance estimates for each of the samples. Because the samples were run in replicate (2–3 per sample), an average peptide abundance and corresponding S.D. was computed for each peptide across the replicates. An abundance estimate for each of the identified open reading frames (ORFs) was computed by averaging the peptide abundance estimates for the ORF using only those peptides whose intensities were ≥33% of the most abundant peptide for the given ORF. More than 200 top hits for peptides found in each of the PSII preparations were scanned for -fold changes across the samples.

Statistical Analysis of Peptide Identification Data—A threshold of 0.18 units was applied to the peptide abundance data to discard all measurements below the chosen noise threshold. Replicate data for each peptide were averaged, and the average abundances were used to calculate log₂ (mutant/wild type) ratios for each peptide. Peptides that were not present in at least two replicates for both wild type and mutant were discarded. The remaining peptides were then used to calculate average log ratios for their respective proteins. S.E. were calculated for the replicates. The results of this proteomics study have been deposited with NCBI under the accession number GSE9577.

Protein Visualization—SDS-PAGE was performed as described previously (19) using a gel with 18–24% acrylamide gradient and 6 M urea. After transfer to 0.22-μm nitrocellulose, PsbO and PsbQ were detected by using specific antisera against each protein, and both were reacted with goat-anti-rabbit horseradish peroxidase-conjugated antisem (Pierce) developed in West Pico (Pierce) for 5 min. PsbV was visualized by reacting its cofactor with SuperSignal West Pico Substrate (Pierce). Blots were visualized in a Fujifilm LAS-1000plus imager (Fujifilm, Stamford, CT) for 1–5 min. Digital images were quantitated using ImageJ software (20).

Reverse Transcription-PCR—Total RNA was isolated from Synechocystis 6803 using TRIZol reagent (Invitrogen) and purified using RNA Clean-Up Kit (Zymo Research, Orange, CA). Single cDNA strands were synthesized (21) using Superscript II control. The cDNA was then treated with RNase H (Invitrogen) and amplified by PCR to create four overlapping cDNA deposits with NCBI under the accession number GSE9577.

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Reverse Transcription-PCR—Total RNA was isolated from Synechocystis 6803 using TRIZol reagent (Invitrogen) and purified using RNA Clean-Up Kit (Zymo Research, Orange, CA). Single cDNA strands were synthesized (21) using Superscript II (Invitrogen). Four primers were used in separate reactions to create cDNA to different overlapping sections of the operon 5’-GGCATGCCAGTGTGTTAGA-3’, 5’-AACAGGGGT-TTAATTTCCCG-3’, 5’-GGTAACACCATGGCCACCT-3’, 5’-TCCTAGGGTCATCTATTTCG-3’). A primer for RNase P (5’-ACCAAAATTCCTCAAGCAGG-3’) provided a positive control. The cDNA was then treated with RNase H (Invitrogen) for 30 min at 37°C and amplified by PCR to create four overlapping products. PCR primers used 5’-GGCATGCCAGTGTGTTAGAT-3’, 5’-TGAGCAACAGTAGAATCTCCCC-3’, 5’-AACAGGGGT-TTAATTTCCCG-3’, 5’-GGTGGAAGGCCCAAAAGC, 5’-GGTAACACCATGGCCACCT-3’, 5’-TGCA-
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CTGATTAGGTTTTTG-3', 5'-TCTTAGGCTCATCTTTCTGC-3', and 5'-CTGCAAAGCCTACTTATTT-3' to amplify slr0144-slr0152 operon and 5'-ACCAATTCCTCA-AAGCG-3' and 5'-CAAAGGTTGCTGGTTAAC-3' to amplify RNase P.

slr0144-slr0152 Deletion Construction—The predicted ORFs of slr0144, slr0145, slr0146, slr0147, slr0148, slr0149, slr0150, slr0151, and slr0152 were replaced by a chloramphenicol resistance gene. The 430 bps upstream of the slr0144 ORF (PCR amplified using primers 5'-AGTCAGGCTCACAAGTT-GGCCGGTCACTCC-3' and 5'-CATGCTATGCCTTTTCAACGGGCAATGCTCTGAA-3') and 460 bps downstream of the slr0152 ORF (PCR-amplified using primers 5'-AGTACGCGTTACTCAGTCA-3' and 5'-GGCTGCGTAATGCTGGTTATG-3' were cloned into flanking positions of the chloramphenicol resistance gene in a pUC18 derivative. The construct was transformed into Synechocystis 6803, and segregation of the mutation was confirmed by PCR. Knock-out construction and segregation of mutant are shown in Fig. 7.

Fluorescence Measurements—Kautsky fluorescence induction and Q<sub>a</sub> reoxidation were measured at room temperature using a FL100 flash kinetic fluorometer (Photon Systems, Brno, Czech Republic) with FluorWin software (Version 3.6.3.3). The Chl concentration for each sample was adjusted to 5 μg Chl/ml as measured on a DW2000 spectrophotometer (SLM-Aminco, Urbana, IL). The samples were dark-adapted for 3 min before measuring.

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State Oxygen Evolution—A Clarke-type electrode was used to determine the rate of photosynthetic oxygen evolution (22). Oxygen evolution was measured for whole cells at a concentration of 5 μg Chl/ml in the presence of 0.5 mM 2,6-di-chloro-p-benzoquinone (Eastman Kodak Co.) and 1 mM K<sub>3</sub>FeCN<sub>6</sub>. Light intensity was adjusted by use of neutral density filters.

Flash Yield Oxygen Evolution and Calculation of S-state Distribution—Flash yield oxygen evolution was measured on a bare platinum electrode (Artisan Scientific Co., Urbana, IL). Cells were incubated in the dark for 2 min before electrode polarization at 0.65 V for 10 s, and a series of 16 flashes was applied. Data points were collected at intervals of 10 μs during the flash train. These data were then analyzed utilizing the in-house software program Oxygen Revolution, and peak data for each sample were fit to a four-step homogenous model of manganese cluster S-state cycling (23). Model-fitting calculations were done using MathCad software (MathSoft Engineering and Education, Inc., Cambridge, MA).

RESULTS

Creation of AMT Tag Library for Synechocystis 6803—To identify novel PSII components, we created an AMT tag library for Synechocystis 6803 (24, 25). Cells were grown under a variety of conditions to induce differential gene expression. Samples for this global approach allows for analysis of mixtures of proteins without initial separation by electrophoresis. The ensuing AMT tag library for Synechocystis 6803 represents ~56% of the proteome.

Identification of Greater than 200 PSII-associated Proteins with Differential Abundance in Lumenal Protein Mutants—To look for assembly intermediates, we compared the protein composition of HT3 PSII, and PSII isolated from the lumenal protein mutants ΔpsbV HT3, ΔpsbQ HT3, and ΔpsbP HT3 (7, 8, 12, 13). The complexes were analyzed using LC/MS, and the component peptides were identified using the AMT tag library. This more sensitive global proteomic technique revealed more than 200 proteins differentially expressed in the lumenal protein mutants as compared with the wild type HT3 (supplemental Table 1). These additional proteins may represent factors that are not part of stable, mature complexes but are associated transiently at some point during the PSII lifecycle, such as assembly, repair, and degradation partners.

Of those identified, 15 were proteins previously identified as PSII-associated proteins (D1, D2, CP43, CP47, PsbE, PsbF, PsbH, PsbL, PsbO, PsbV, PsbU, PsbQ, Psb27, Psb28, and Psb29). To confirm the validity of this proteomic analysis, we compared levels of PSII stoichiometric components as determined by this proteomics analysis and by established immunological methods. Previous studies have shown that loss of any of the lumenal proteins results in a destabilization of the entire luminal face of PSII and correlates with reduced levels of the other lumenal proteins (8, 27–29). Levels of PsbO, PsbQ, and PsbV in the strains HT3, ΔpsbV HT3, ΔpsbQ HT3, and ΔpsbP HT3 were analyzed by comparative immunoblotting experiments. These experiments demonstrated the decrease of additional luminal proteins in the mutants as compared with the HT3 strain. The band intensities were calculated using ImageJ (see supplemental Fig. S1 for linearity of band intensities across proteins concentrations).

Comparison of the ratios of PsbO, PsbQ, and PsbV levels in the strains HT3, ΔpsbV HT3, ΔpsbQ HT3, and ΔpsbP HT3 observed from the AMT tag analysis to ratios determined using immunological assays showed that the data from both detection methods closely corresponded, confirming the AMT tag methodology for quantification of protein abundances (Fig. 2).

Previously, the interactions of the extrinsic proteins have been analyzed by the genetic deletion or affinity tagging of individual PSII subunit and analyzing the corresponding changes in the polypeptide profiles of the complex. In contrast, this high throughput approach provides a comprehensive data set to analyze PSII interactions. For instance, analysis of PsbQ using affinity tagging has shown that PsbQ is found solely in fully assembled complexes (8). Correspondingly levels of PsbQ in complexes from ΔpsbV HT3 and ΔpsbP HT3 were observed from the AMT tag analysis to ratios determined using immunological assays showed that the data from both detection methods closely corresponded, confirming the AMT tag methodology for quantification of protein abundances (Fig. 2).

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decrease in the absence of psbQ (28), which corresponds to decreased levels of PsbV seen in all mutants in this study (Fig. 3). Psb28 and Psb29 are conserved among a variety of photosynthetic organisms, and although they have been shown to display PSII association and function (32–34), their functions have not been elucidated. Thus, it is intriguing that Psb28 increases significantly in abundance in all three mutants and Psb29 increases markedly in ΔpsbQ HT3 and ΔpsbP HT3 and decreases in ΔpsbV HT3.

Of the 200 proteins initially identified, 50 were proteins of unknown function whose levels were altered significantly in the mutants as compared with HT3 (supplemental Table 1). Although a concomitant loss of additional proteins with the deletion of PsbP, PsbQ, and PsbV is well documented (8, 26–28), the large group of proteins that increased in abundance as a result of these deletions is intriguing. This group of proteins may represent assembly and degradation factors associated with the lifecycle-arrested complexes.

Proteins in a Single Genic Cluster Have Increased Abundance in ΔpsbV HT3, ΔpsbQ HT3, and ΔpsbP HT3 PSII Complexes—Analysis of the proteins that increased the most in the mutant PSII complexes revealed 4 proteins, Slr0146, Slr0147, Slr0149, and Slr0151, which were up to 12.5 times more abundant in the mutant PSII complexes. A closer look at the context of these proteins revealed that their ORFs are located within the same genic context in the Synechocystis genome (Fig. 4A).

Previously published microarray experiments describe this gene cluster, slr0144-slr0152, as one of the most highly coordinated in Synechocystis and reveal that transcripts are down-regulated in cells experiencing oxidative stress due to low iron or treatment with hydrogen peroxide (35). Additionally, expression of these genes is down-regulated in mutants lacking PSI or PSI and phycobilisome proteins (35). This suggests that the coordinated increase in protein levels observed in this study is not a general stress response and is specific to PSII function. Reverse transcription-PCR experiments confirmed that the ORFs slr0144-slr0152 are located on a single transcript and, thus, are in an operon (data not shown).

It is important to note that Slr0144 and Slr0145 were also observed in the isolated PSII samples in several replicates, but

FIGURE 2. Comparison of protein quantification determined by proteomic and immunoblot analysis. A, levels of PsbO (white bars), PsbQ (black bars), and PsbV (hatch bars) as determined by proteomics analysis. B, Western blot analysis of PsbO, PsbQ, and PsbV. C, comparison of PsbO, PsbQ, and PsbV levels as determined from proteomic and from immunoblot analysis, as quantified by ImageJ software.

FIGURE 3. Levels of PSII extrinsic proteins in ΔpsbP HT3, ΔpsbQ HT3, and ΔpsbV HT3. Levels are determined as the log2 ratio of ion counts for the corresponding protein in ΔpsbP HT3 (white bars), ΔpsbQ HT3 (black bars), and ΔpsbV HT3 (hashed bars) PSII as compared with HT3 PSII. Error bars represent the indicated S.E.
Slr0144 and Slr0147 both contain a 4-vinyl reductase domain (35, 38). This domain is predicted to be a small molecule binding domain, and a protein containing this domain has been shown to be involved in chlorophyll biosynthesis in *Rhodobacter capsulatus* (39). These two proteins contain the only two 4-vinyl reductase domains found in *Synechocystis* 6803. Slr0148 and Slr0150 are putative ferredoxins and contain motifs for 2Fe-2S iron-sulfur clusters, cofactors that mediate electron transfer and are found in the cytochrome b$_6$f complex and the PSI-associated terminal ferredoxin (35, 38). Additionally, Slr0146 and Slr0149 have domains for the binding of bilins, cofactors involved in photosynthesis (38). Slr0151 contains a tetratricopeptide repeat domain hypothesized to be involved in protein-protein interactions, suggesting that these proteins may complex with each other or with as yet unidentified partners (35, 38). Slr0152, also named PknD, encodes a Ser/Thr kinase, which may function as a regulatory element for the operon (35, 38).

**Deletion of slr0144-slr0152 Does Not Alter Photoautotrophic Growth**—To investigate the role of the slr0144-slr0152 operon in PSII function, the entire coding region of the operon was deleted and replaced with a chloramphenicol resistance gene (Fig. 6). Deletion of the operon did not alter photosynthetic growth (Fig. 7A). Depletion of CaCl$_2$ from the culture media did not affect the rate of photoautotrophic growth, as seen in other PSII extrinsic mutants (data not shown). Growth of the mutant in high (100 μmol photons m$^{-2}$ s$^{-1}$) or low (20 μmol photons m$^{-2}$ s$^{-1}$) light conditions also showed no difference from wild type (data not shown).

**The Operon Contains Binding Domains for Cofactors Important in Photosynthesis**—Although the function of these proteins is unknown, their sequences provide interesting insights into possible roles in photosynthesis. All nine proteins are predicted to be located in the cytoplasm (36, 37). Many of these proteins contain binding motifs for cofactors involved in photosynthesis as well as regulatory elements (Fig. 5). Slr0144 and Slr0147 both contain a 4-vinyl reductase domain (35, 38). This domain is predicted to be a small molecule binding domain, and a protein containing this domain has been shown to be involved in chlorophyll biosynthesis in *Rhodobacter capsulatus* (39). These two proteins contain the only two 4-vinyl reductase domains found in *Synechocystis* 6803. Slr0148 and Slr0150 are putative ferredoxins and contain motifs for 2Fe-2S iron-sulfur clusters, cofactors that mediate electron transfer and are found in the cytochrome b$_6$f complex and the PSI-associated terminal ferredoxin (35, 38). Additionally, Slr0146 and Slr0149 have domains for the binding of bilins, cofactors of the phycobilisome, the light harvesting system of cyanobacteria (38). Slr0151 contains a tetratricopeptide repeat domain hypothesized to be involved in protein-protein interactions, suggesting that these proteins may complex with each other or with as yet unidentified partners (35, 38). Slr0152, also named PknD, encodes a Ser/Thr kinase, which may function as a regulatory element for the operon (35, 38).

**Deletion of slr0144-slr0152 Mutant Displays Normal Fluorescence Kinetics**—Fluorescence measurements with the deletion mutant did not exhibit any defects in PSII electron transfer.
Measurements of fluorescence induction (Kautsky effect) show no differences among values for \( F_o \), \( F_w \), and \( F_v \) (data not shown). Similarly, measurements of \( Q_A \) reoxidation using the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea also showed in no differences in these fluorescence parameters. This indicates that electron flow through the core of PSII is largely unaffected by the deletion of \( slr0144-slr0152 \).

Proteomics of PSII Assembly

The \( \Delta slr0144-slr0152 \) Mutant Displays Altered Water Oxidation—Careful analysis revealed that the \( \Delta slr0144-slr0152 \) mutant has impaired oxygen evolution activity (Fig. 7B). The mutant produces only ~80% as much oxygen as wild type, indicating an impairment of PSII complexes upon deletion of the \( slr0144-slr0152 \) operon. Further analysis of oxygen evolution by measuring flash oxygen yield showed a four-period oscillation similar to wild type but with decreased yield in the \( \Delta slr0144-slr0152 \) mutant (Fig. 7C). Quantification of this data revealed that before illumination, \( \Delta slr0144-slr0152 \) shows no significant change in the percentage of centers at the \( S_0 \) state but has a decreased percentage of reaction centers in the \( S_1 \) state and increased percentages of centers in the \( S_2 \) and \( S_3 \) states. A similar increase in \( S_2 \) stabilization is seen in the \( \Delta psbV \) mutants (40) (Fig. 7D). Based on these data, we have named the products of the \( slr0144-slr0152 \) operon Photosystem II (Pap).

DISCUSSION

Identification of Novel PSII-associated Proteins—Using a sensitive proteomic approach to investigate the composition of PSII, we identified more than 200 proteins associated with the complex, 169 more proteins than had been identified using more conventional methods. Additionally, the small number of PSI and PSII-associated proteins that were identified in the proteomics study (5% of the total identified proteins) suggest that the PSII samples were relatively pure and that the majority of the proteins identified are indeed PSII associated. These additional proteins may represent factors that are transiently associated with PSII and play roles in complex assembly, repair, or degradation. In the past, it has proven difficult to identify these proteins due to the relatively short periods of association in comparison to mature, active PSII complexes. This type of proteomics analysis is a valuable tool in investigating proteins involved in the complex assembly of PSII.

Identification of a Novel Operon Involved in PSII Activity—Using established histochemical methods, we were able to con-
firm that this proteomic approach is an accurate method to establish quantitative protein profiles in isolated PSII complexes. Of the 50 PSII-associated proteins identified with unknown functions, 4 of the proteins that were increased in abundance in the PSII mutants were located in a single genic region. These proteins are encoded in a nine-gene operon known to be highly coordinated transcriptionally, and at least six operon products co-purify with PSII complexes. Additionally, the slr0144-slr0152 operon contains domains for binding the cofactors chlorophyll, 2Fe–2S iron sulfur centers, and bilin, which are all components of the electron transport chain.

The conservation of synteny of the operon along with the diversity of the cyanobacterial species (mesophiles, thermophiles, and nitrogen fixers) in which the genes of the slr0144-slr0152 operon has been observed suggests that these genes play an important role in cellular processes. Thus, it is intriguing that only two of the genes, slr0150 and slr0152, are conserved in higher photosynthetic organisms (41), suggesting that the mode of this action is specific to cyanobacteria.

It has long been known that genes in bacterial operons undergo coordinated transcriptional regulation. However, it is unclear whether those transcripts then undergo coordinated translation and assembly into a complex, although there are examples such as the ribosomal operons. Because the Slr0144, Slr0145, Slr0146, Slr0147, Slr0149, and Slr0151 proteins co-purified with PSII complexes under stringent conditions, it appears that not only is transcription of this operon tightly regulated, as shown by Singh et al. (35) but also that the transcripts undergo coordinated translation and bind to the same complex.

Role of Paps in PSII Assembly and Function—Because there is no change in photautotrophic growth of the Δslr0144-slr0152 mutant in any of the conditions tested, this suggests that the role of slr0144-slr0152 is nonessential and is primarily involved in increasing photosynthetic efficiency. Additionally, the lack of altered fluorescence kinetics suggests that the core complex of PSII is fully assembled in Δslr0144-slr0152. However, the decrease in PSII activity suggests that with the loss of the Paps there is a decreased number of fully assembled PSII complexes. Alternatively, this decreased PSII activity could also be due to complexes that are fully assembled but are somehow impaired for photochemistry.

The Δslr0144-slr0152 mutation demonstrates a case of cross-talk between the luminal and cytosolic proteins of PSII. It is intriguing that deletion of the luminal proteins PsbP, PsbQ, and PsbV led to an increased in the abundance in the cytosolic Pap proteins. The increase of these proteins in non-fully assembled PSII complexes suggests that the Pap proteins function in assembly of complexes and are aggregating on these sub-assembled complexes or are functioning in degrading the non-fully functional complexes. Additionally, the decrease in oxygen evolution activity and the altered S state distribution in the Δslr0144-slr0152 mutant are phenotypes traditionally associated with mutation in the luminal PsbO, PsbU, PsbV, PsbQ, and PsbP proteins (7, 28, 40), suggesting that the luminal side of the complex is unstable in the absence of the Pap proteins. This suggests that there is feedback across the membrane plane of PSII that increases Pap protein levels when the luminal surface is non-fully assembled and that the Pap proteins are necessary to fully assemble the luminal side of PSII.

PSII has an intricate lifecycle and the rudimentary steps of assembly have been elucidated. However the assembly of the proteins and cofactors are not yet fully understood. The discovery of Paps may aid our understanding of how the non-protein cofactors are inserted or recycled into new and repaired complexes. It is possible that the PSI defects seen in this study indicate that Slr0144 and Slr0147 function to sequester chlorophyll molecules to prevent damage to the cell before initial complex assembly and during repair, similar to the small CAB-like proteins (11). Future work demonstrating that the cofactor binding sites of the Pap proteins are functional and that they are able to transfer cofactors could provide exciting insight into how these cofactors are assembled into the complex. Additionally, Pap proteins shed new light on the cross-talk that must occur between the cytosolic and luminal compartments of the cyanobacterial cell.

In addition, future work will need to focus on whether Paps are key to PSII assembly or if they aid in assembly of other complexes in the electron transport chain. Because the operon contains binding domains for 2Fe–2S clusters and bilin, cofactors of cytochrome b/f complex, ferredoxins, and the phycobilisome in addition to domains for chlorophyll binding, which is integral to both PSI and PSII function, Pap must serve as a cofactor repository for the entire photosynthetic chain. Preliminary data suggest that the deletion of slr0144-slr0152 does not affect the abundance or connectivity of PSI or the phycobilisome, perhaps because these systems are less sensitive to the affects of the removal of Pap proteins on than PSII. However, it is clear from this study that these proteins play an important role in PSII.

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