Proteomic Analysis of a Highly Active Photosystem II Preparation from the Cyanobacterium *Synechocystis* sp. PCC 6803 Reveals the Presence of Novel Polypeptides†

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ABSTRACT: A highly active oxygen-evolving photosystem II (PSII) complex was purified from the HT-3 strain of the widely used cyanobacterium *Synechocystis* sp. PCC 6803, in which the CP47 polypeptide has been genetically engineered to contain a polyhistidine tag at its carboxyl terminus [Bricker, T. M., Morvant, J., Masri, N., Sutton, H. M., and Frankel, L. K. (1998) *Biochim. Biophys. Acta 1409*, 50−57]. These purified PSII centers had four manganese atoms, one calcium atom, and two cytochrome b_{559} hemes each. Optical absorption and fluorescence emission spectroscopy as well as western immunoblot analysis demonstrated that the purified PSII preparation was devoid of any contamination with photosystem I and phycobiliproteins. A comprehensive proteomic analysis using a system designed to enhance resolution of low-molecular-weight polypeptides, followed by MALDI mass spectrometry and N-terminal amino acid sequencing, identified 31 distinct polypeptides in this PSII preparation. We propose a new nomenclature for the polypeptide components of PSII identified after PsbZ, which proceeds sequentially from Psb27. During this study, the polypeptides PsbJ, PsbM, PsbX, PsbY, PsbZ, Psb27, and Psb28 proteins were detected for the first time in a purified PSII complex from *Synechocystis* 6803. Five novel polypeptides were also identified in this preparation. They included the Sll1638 protein, which shares significant sequence similarity to PsbQ, a peripheral protein of PSII that was previously thought to be present only in chloroplasts. This work describes newly identified proteins in a highly purified cyanobacterial PSII preparation that is being widely used to investigate the structure, function, and biogenesis of this photosystem.

During the photosynthetic light reactions in the thylakoid membranes of cyanobacteria, algae, and plants, photosystem II (PSII), a multisubunit membrane protein complex, catalyzes oxidation of water to molecular oxygen and reduction of plastoquinone (*1*, *2*). Extensive biochemical and genetic studies have shown that the PSII complex is composed of a large number (>15) of polypeptides (*3*−*5*). A comparison of different purified PSII complexes reveals notable differences in the polypeptide compositions between preparations. Such differences are due to several factors, including advancements in purification of membrane bound complexes and improved techniques for polypeptide analysis. In addition, the protein composition of PSII from prokaryotic cyanobacteria differs significantly from that of PSII from plant and algal chloroplasts (*5*). For example, cytochrome (cyt) c_{550} (PsbV) and PsbU, two extrinsic proteins, are present in cyanobacterial PSII but are absent from PSII in plants and green algae (*6*, *7*). Shen, Inoue, and co-workers have proposed that among the PSII proteins, cyt c_{550} and PsbU in cyanobacteria function in place of the 24 and 17 kDa extrinsic polypeptides, PsbP and PsbQ, in chloroplasts in green algae and plants (*6*).

Recently, Zouni et al. made a great step forward by determining the structure of a PSII complex isolated from the thermophilic cyanobacterium, *Synechococcus elongatus*, by X-ray crystallography (*1*). This work revealed the relative positions of the major transmembrane helices in PSII, as well as the relative positions of a number of important cofactors. However, at the current 3.8 Å resolution, important details of the structure of this large protein complex remain undefined, including the precise locations of many of the polypeptide components.

The mesophilic cyanobacterium *Synechocystis* sp. PCC 6803 has been widely used for genetic and biochemical analysis of the PSII complex (*2*, *8*). During recent years, Bricker and co-workers (*9*), as well as Brudvig, Diner, and their colleagues (*10*, *11*), have genetically modified the *psbB*

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gene in this organism so that the encoded CP47 protein has a hexahistidyl tag at its carboxyl end. The presence of this polyhistidine tag allows simple and rapid purification of PSII with high oxygen evolution activity. Such purified complexes are currently being used in a number of laboratories for spectroscopic analysis of PSII from *Synechocystis* 6803 (*11*−*14*). Over the past few years, the addition of a polyhistidine tag has been exploited to purify a number of membrane protein complexes such as the bacterial reaction center (*15*), cytochrome *c* oxidase (*16*), cytochrome bo_3 (*17*), and cytochrome bc_1 (*18*) complexes from *Rhodobacter sphaeroides*, as well as PSII from thermophilic cyanobacteria (*19*) and the green alga *Chlamydomonas reinhardii* (*20*), in highly active forms.

In anticipation of higher-resolution structural information about PSII, it is important to know the identities of all of the polypeptide components of this large protein complex. In an earlier study, Ikeuchi and co-workers used an SDS−PAGE system (*21*) designed to resolve small proteins to identify several previously unknown PSII proteins, including PsbI (*21*) and PsbM (*22*). Over the past decade, additional low-molecular-mass PSII polypeptides have been identified (*3*−*5*, *8*). During the present study, we have purified a His-tagged PSII preparation from *Synechocystis* 6803 and performed a detailed analysis of the complex. A number of assays demonstrated that this PSII preparation is extremely pure and has high activities. We have also determined the contents of several important pigments and metal cofactors in this isolated complex. Finally, the PSII polypeptides were fractionated using an improved SDS−PAGE system designed for the analysis of membrane protein complexes (*23*) and were examined using mass spectrometry that allows identification of polypeptides even when their N-termini are blocked. These proteomic technologies, together with the availability of the complete genome sequence of *Synechocystis* 6803 (*24*), resulted in a comprehensive analysis of all of the detectable polypeptide components in this purified PSII preparation. A number of proteins, such as PsbJ and PsbY, were found for the first time in a cyanobacterial PSII complex. A major finding of this study was the identification of five novel polypeptides in cyanobacterial PSII complex, including a homologue of the PsbQ protein, which was previously thought to be present only in chloroplasts.

EXPERIMENTAL PROCEDURES

Cyanobacterial Culture Conditions. The HT-3 strain (*9*) of *Synechocystis* 6803 with a hexahistidyl tag at the C-terminal end of the CP47 protein was a generous gift from Prof. T. M. Bricker (Louisiana State University). HT-3 cells were grown in liquid BG11 medium (*25*) supplemented with 2 mM glucose and 50 μM kanamycin, with vigorous bubbling with air, under 50 μmol photons m^{-2} s^{-1} at 30 °C.

Purification of PSII. Cells were harvested and washed with Buffer A [50 mM MES−NaOH (pH 6.0), 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 25% glycerol] and resuspended in the same buffer. Concentrated cells were frozen and stored at −80 °C, which facilitated breakage of cells. PSII complexes were purified as described in ref *9* with the following modifications. After thawing, cells were broken with 0.17 mm glass beads using 24 break cycles, each cycle consisting of 10 s of homogenization, followed by 2 min of cooling, using a prechilled Bead-Beater chamber (Bio-Spec Products, OK). Cell homogenates were centrifuged at 37 000*g* for 15 min and washed once. The resulting pellet was resuspended in Buffer A at 1 mg chlorophyll (Chl[1])/mL. Dodecyl maltoside (Anatrace, OH) was added from a 20% (w/v) solution to a final concentration of 0.8%. After incubation with gentle stirring for 20 min at 4 °C, the sample was centrifuged at 37 000*g* for 10 min. The resulting supernatant was loaded on an Ni−NTA column (Qiagen) pre-equilibrated with Buffer A that was supplemented with 0.04% dodecyl maltoside. The column was washed with the same buffer, after which bound His-tagged PSII complexes were eluted using Buffer A supplemented with 50 mM histidine and 0.04% dodecyl maltoside. After the addition of 0.8 volume of 25% poly(ethylene glycol) 8000 in 30 mM MES−NaOH (pH 6.0), the PSII material was immediately precipitated by centrifugation at 37 000*g* for 10 min.

Salt Treatment. Purified PSII at 1 mg Chl/mL was incubated with 1 M $CaCl_2$ or 1 M Tris−HCl (pH 8.0) for 30 min on ice in the dark (*26*). After centrifugation at 360 000*g*, the precipitated PSII complexes were washed once and finally resuspended in Buffer A.

Gel Filtration. For size-separation chromatography, purified PSII complexes were fractionated on Sephadex S-400 HR in a XK16/70 column (50 cm, Amersham Pharmacia Biotech), pre-equilibrated with a solution containing 50 mM MES−NaOH (pH 6.5) and 150 mM NaCl, at a flow rate of 0.1 mL/min. The molecular mass of the PSII complex was estimated using a gel filtration calibration kit (Amersham Pharmacia Biotech). Absorbance of polypeptides was recorded at 280 nm on an SPD-6AV UV−vis spectral detector (Shimadzu, Kyoto).

SDS−PAGE, Heme Staining, and Immunoblot Analysis. Polypeptides were resolved according to ref *23*. This procedure allows optimal resolution of small proteins without sacrificing the resolution in high-molecular weight regions. Samples were loaded on 18−24% gradient acrylamide gels containing 6 M urea, and the fractionated polypeptides were stained with Coomassie blue. For heme staining, the gels were soaked in water for 20 min, and then incubated in a solution containing 0.2% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (DAB), 25% methanol, and 0.25 M sodium citrate (pH 4.7) for 1 h. 2% hydrogen peroxide was then added for color development. Western Immunoblot analysis was performed as described in (*27*).

N-Terminal Amino Acid Sequencing. N-terminal sequences of polypeptides were determined on a protein sequencer (model 473A, Applied Biosystems) as described in ref *23*. The polypeptides were identified using the CyanoBase genome database for *Synechocystis* 6803 (*28*).

MALDI Mass Spectrometry. Individual polypeptides separated by SDS−PAGE, visualized using Coomassie staining were excised, and subjected to in-gel digestion with trypsin. The extracted peptides were analyzed by matrix-assisted laser desorption ionization mass spectroscopy (MALDI), either

[1] Abbreviations: Chl, chlorophyll; cyt, cytochrome; DAB, 3,3′-diaminobenzidine tetra-hydrochloride; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FeCN, potassium ferricyanide; MALDI, matrix-assisted laser desorption ionization mass spectroscopy; MSP, manganese stabilizing protein; Pheo, pheophytin; PSI and PSII, photosystems I and II, respectively; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

directly or after purification using a reversed-phase microcolumn (Zip-Tip, Millipore). MALDI was performed on a Voyager DE-STR instrument (PerSeptive Biosystems, Inc. Framingham, MA) using α-cyano-4-hydroxycinnamic acid as the matrix. Identities of some of the proteins were confirmed by further using nanoelectrospray tandem mass spectrometry on a Q-TOF mass spectrometer (Micromass, Inc. Beverly, MA). The Protein Prospector suite of programs (*29*) was used to search the *Synechocystis* 6803 protein sequence database, which was downloaded from the CyanoBase website (*28*). Many of the small (<10 kDa) polypeptides could not be unambiguously identified using mass spectrometry. This is due to the fact that small polypeptides generate very few tryptic peptides so that there may be only one or two peptides observed in the mass spectrum.

Absorption and Fluorescence Spectroscopy. To estimate cyt b_{559} content, we obtained reduced-minus-oxidized difference absorption spectra on a DW2000 spectrophotometer (SLM-Aminco, Urbana, IL). The concentration of cyt b_{559} was determined using a reduced minus oxidized extinction coefficient of 17.5 mM^{-1} cm^{-1} for the wavelength pairs 559 and 570 nm (*30*). Fluorescence emission spectra at 77 K were measured on a Fluoromax-2 fluorometer (Jobin Yvon, Cedex, France) (*27*). Samples were suspended in Buffer A supplemented with 0.04% dodecyl maltoside. The concentration of Q_A was determined using the method described in ref *31*.

Metal Content. The contents of bound calcium, manganese, and iron were determined using a flameless atomic absorption spectrometer (AA-660G, Shimadzu, Kyoto) equipped with a GFA-4B graphite furnace atomizer (*32*).

Pigment Analysis. The concentrations of Chl were determined according to ref *33*. The molar ratio of Chl to pheophytin (Pheo) was determined by HPLC as described in (*34*). The concentrations of β-carotene and zeaxanthin were determined according to (*34*). The Chl/P700 ratio was determined from ascorbate-reduced minus ferricyanide-oxidized chemical difference spectra as described in ref *35*.

Oxygen Evolution. Steady-state rates of oxygen evolution were measured at 30 °C using a Clark-type electrode in combination with an OxyCorder 301S recording device and OxyWin Software (Photon System Instruments, Brno, Czech Republic). PSII samples were suspended at 2 μg Chl mL^{-1} in Buffer B [50 mM MES−NaOH (pH 6.0), 10 mM $MgCl_2$, 5 mM NaCl, and 0.5 M sucrose] to which 2,6-dichloro-*p*-benzoquinone (DCBQ) and potassium ferricyanide or duroquinone were added as electron acceptors.

RESULTS

Protein Composition of the Purified PSII Complex. Figure 1 shows the polypeptide profiles of solubilized thylakoid membranes (lane A) and purified PSII complexes (lane B). The membrane sample contained a large amount of phycobiliproteins (~16 kDa). These predominant polypeptides were completely removed during the purification of the His-tagged PSII complex (lane B), indicating that the simple isolation scheme has yielded a highly pure fraction (*9*). In the purified PSII complex, more than 30 distinct polypeptide bands were detected, ranging from ~3 to ~100 kDa (Figure 1, lane B). Each of the polypeptides numbered 1−36 in lane B was subjected to N-terminal sequencing (Table 1). Among the larger polypeptides, the following known PSII components were identified: CP47 (PsbB), the manganese-stabilizing protein (MSP or PsbO), the large subunit of cytochrome b_{559} (PsbE), cyt c_{550} (PsbV), Psb28[2] (Sll1398; also known as Ycf79), and PsbU (*2*, *5*). In addition, we detected 10 distinct low-molecular weight (<10 kDa) polypeptides: PsbH, PsbF, PsbI, PsbL, $PsbT_c$, PsbJ, PsbM, PsbX, PsbK, and PsbY (Table 2). We also identified PsbZ (Sll1281, Ycf9), a polypeptide that has recently been described to have a role in the dimerization of PSII in tobacco and *Chlamydomonas* chloroplasts (*36*). Finally, two novel polypeptides, the products of the *sll1414* and *sll1130* genes (Table 2), were also detected.

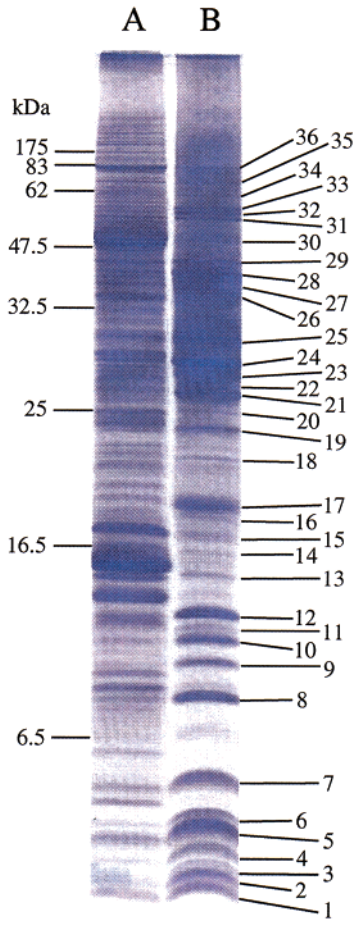

FIGURE 1: Polypeptide profiles of detergent-solubilized membrane and purified His-tagged PSII complex from the HT-3 strain of *Synechocystis* 6803. A, membrane; B, purified PSII complex. Numbers on the right correspond to those in Table 1. The positions of molecular-weight standards are shown on the left. 10 μg Chl-containing sample was applied to each lane, fractionated by SDS−PAGE, and the polypeptides were stained with Coomassie blue.

As shown in Table 1, the identities of a number of polypeptides (Figure 1) could not be determined by N-terminal sequencing, presumably because the N-termini of these proteins were blocked. In a complementary approach, all of the Coomassie-stained and >10 kDa polypeptides were subjected to MALDI mass spectroscopic analysis. Most of the polypeptides identified by N-terminal sequencing were also identified by MALDI peptide mass mapping (Table 1). In addition, this latter technique revealed the presence of D1 (PsbA) and D2 (PsbD), the reaction center proteins of PSII, as well as CP43 (PsbC) and Psb27[2] (Slr1645) in this PSII preparation. We also identified two different isoforms of FtsH, a protease that has recently been shown to have a role in the degradation of the rapidly turned over D1 protein

Table 1: Polypeptide Profile of Purified PSII Complex from *Synechocystis* 6803. Proteins Were Identified on the Basis of N-Terminal Sequence and Mass Spectroscopic (MS) Data[a]

polypeptide no.	N-terminal sequence	identity by sequence	identity by MS
1	MDwRV IVVVS PLLIA	PsbY	n.d.
	KLPEA YQIFD PLVDV	PsbK	n.d.
2	MTPSL ANFL	PsbX	n.d.
	MQVNN LGFI	PsbM	n.d.
3	MFAEG RIPLWVV	PsbJ	n.d.
4	MESVA YILVL	$PsbT_c$	n.d.
5	MDRNS NPNRQ PVELN RTSLY	PsbL	n.d.
	MLTLK IAV	PsbI	n.d.
	ATQNP NQP	PsbF	n.d.
6	MSIVF QIALA ALV	PsbZ	n.d.
	ATQNP NQPxx YPI	PsbF	n.d.
7	AQRTR LGDIL RPLNS EY	PsbH	Ssl2598 (PsbH)
8	SGTTG ERPFS D	PsbE	Ssr3451 (PsbE)
9	(−)		Slr1645 (Psb27)[b]
10	ELNAV DAKLT TDFGQK	PsbU	Sll1194 (PsbU)
11	MNTIY EQFDV VIVPVP	Sll1130	n.d.
	AEIQF SKGVA E	Psb28[b]	
12	(−)		Sll1638
13	(−)		n.d.
14	(−)		n.d.
15	(−)		Slr0172 (GuaB)
16	VELTE STRTI PLDE	PsbV	Sll0258 (PsbV)
17	VELTE STRTI PLDE	PsbV	Sll0258 (PsbV)
18	(−)		Sll1390
19	xKIRT VSDAK RKFF	Sll1414	Sll1252
20	(−)		Sll1867 (PsbA3)/Slr0427 (PsbA2)
21	(−)		Sll1867 (PsbA3)/Slr0427 (PsbA2)
22	VDKSQ L	MSP	Sll0427 (MSP)
23	(−)		Sll0849 (PsbD1)/Slr0927 (PsbD2)
24	VDKSQ LTYDD I	MSP	Sll0427 (MSP)
25	(−)		Sll0851 (PsbC)
26	(−)		Sll0401 (GltA)
			Slr0906 (PsbB)
27	GLPWY RVHTV	PsbB	Slr0906 (PsbB)
28	GLPWY RVHTV V	PsbB	Slr0906 (PsbB)
29	(−)		n.d.
30	(−)		Sll0258 (PsbV)
			Slr0906 (PsbB)
			Slr1550 (LysS)
31	(−)		Slr1604 (FtsH)
32	(−)		n.d.
33	mKFSx RTALL xsLP	FtsH	Slr0228 (FtsH)
34	(−)		Sll1867 (PsbA3) + sll0849 (PsbD1)
35	(−)		n.d.
36	(−)		n.d.

[a] The polypeptide numbers correspond to Figure 1. Polypeptides whose N-termini were blocked or with weak signals are shown as (−). x, unidentified amino acid residue; lowercase letter in N-terminal sequences, ambiguous but plausible amino acid residue; n.d., not determined. The identities by MS were according to the Cyanobase database (*28*). See Results for further details. [b] New nomenclature.[2]

(*37*), as well as three presumably contaminating proteins, LysS, GltA, and GuaB (Table 2). Finally, mass spectrometric analysis revealed the presence of three additional novel polypeptides, Sll1252, Sll1390, and Sll1638.

In summary, use of two complementary techniques has identified 31 distinct proteins in this isolated PSII complex. However, six proteins, corresponding to bands 13, 14, 29, 32, 35, and 36 (Figure 1 and Table 1), remained unidentified in this analysis. To our knowledge, this is the first report in which all of the known cyanobacterial PSII proteins, except PsbN (*1*), have been identified in a single purified PSII preparation. In this context, it is noteworthy that recent analysis of the polypeptide composition of a PSII preparation from the thermophilic cyanobacterium *Synechococcus vulcanus* has shown that the protein which was originally identified as PsbN in such a preparation (*22*) is actually $PsbT_c$ (Kashino, Y., Koike, H., Yoshio, M., Egashira, H., Ikeuchi, M., Pakrasi, H. B. and Satoh, K., manuscript in preparation), and it is highly likely that PsbN is not a component of PSII.

Among the novel proteins, the amount of the Sll1638 protein (Figure 1, lane B, band 12) was comparable to that of the large subunit of cyt b_{559} (Figure 1, lane B, band 8), eliminating the possibility that it is a minor contaminating protein present in a substoichiometric amount. Notably, BLAST analysis using the GENBANK database revealed that Sll1638 exhibited significant sequence similarity (32% identity; 51% similarity) with the "17 kDa" PsbQ protein

[2] Suggested nomenclature for newly identified PSII proteins and their genes. Conventionally, the genes encoding various PSII proteins have been designated as *psbA*, *psbB*, etc. (*8*). As discussed in this manuscript, more than 26 distinctly different polypeptides (PsbA to PsbZ, with the exception of PsbG) have been identified in PSII in cyanobacteria and plants (*5*). After consultation with various experts in the field, we are proposing that after *psbZ*, the newly identified genes are named *psb27*, *psb28*, etc., and the corresponding proteins are called Psb27, Psb28, etc. A similar nomenclature has been successfully used for the components of ribosome, another large mutiprotein complex.

Table 2: Summary of Polypeptide Composition of Purified His-Tagged PSII Complex[a]

protein (gene)	M_r (kDa)
polypeptides that are known to be associated with PSII	
1. CP47 (*psbB*)	45
2. CP43 (*psbC*)	34
3. Mn-stabilizing protein MSP (*psbO*)	31
4. D2 (*psbD1*, *psbD2*)	29
5. D1 (*psbA2*, *psbA3*)	27
6. cytochrome c_{550} (*psbV*)	16
7. Psb28[b] (*sll1398*)	10
8. PsbU (*psbU*)	10
9. Psb27[b] (*slr1645*)	9.1
10. cytochrome b_{559} large subunit (*psbE*)	7.8
11. PsbH (*psbH*)	5.7
12. PsbZ[b] (*ycf9*, *sll1281*)	4.9
13. cytochrome b_{559} small subunit (*psbF*)	4.9
14. PsbI (*psbI*)	4.6
15. PsbL (*psbL*)	4.6
16. $PsbT_c$[b] (*smr0001*)	4.2
17. PsbJ (*psbJ*)	4.0
18. PsbM (*psbM*)	3.8
19. PsbX (*psbX*)	3.8
20. PsbK (*psbK*)	3.6
21. PsbY (*psbY*)	3.6
other polypeptides with known functions	
22. FtsH protease (*slr0228*)	59
23. FtsH protease (*slr1604*)	57
24. lysyl-tRNA synthetase (*lysS*, *slr1550*)	51
25. citrate synthase (*gltA*, *sll0401*)	42
26. IMP dehydrogenase (*guaB*, *slr0172*)	15
novel polypeptides	
BLAST2 homology	
27. Sll1414 ORF in *Arabidopsis*	24
28. Sll1252 ORF in *Arabidopsis*	24
29. Sll1390 ORF in *Arabidopsis*	21
30. Sll1638 PS II O_2-evolving complex protein 3 (PsbQ) in *Arabidopsis*	11
31. Sll1130 ORF in *Anabaena* 7120	10

[a] M_r, apparent molecular mass in Figure 1. The gene names are from the Cyanobase database (*28*) [b] New nomenclature.[2]

from the vascular plant *Arabidopsis thaliana*. As shown in Table 2, three additional novel polypeptides, Sll1414, Sll1252 and Sll1390 have strong homologues in *Arabidopsis*. Moreover, each of these plant proteins is predicted to have a transit peptide for chloroplasts, suggesting that they are localized in this organelle, consistent with their predicted association with PSII. The fifth novel protein Sll1130 has a significant homologue in the filamentous cyanobacterium *Anabaena* sp. PCC 7120, whose complete genome sequence has been determined recently (*38*). It is noteworthy that other than PsbQ, functions of all of these homologues are currently unknown.

As discussed earlier, PsbQ is a lumen-localized component of PSII in plants and green algae and is known to dissociate from the protein complex upon incubation with high concentrations of $CaCl_2$ as well as Tris (*39*). Hence, salt washing of the purified cyanobacterial PSII complex was carried out to examine whether the Sll1638 protein is also dissociated from PSII under similar conditions. Five polypeptides were removed by both $CaCl_2$ and Tris washing (Figure 2). The major ones were MSP, cyt c_{550}, and PsbU, a finding similar to that described by Shen and co-workers (*6*). Among the released polypeptides, only cyt c_{550} reacted positively to heme staining (Figure 2). One of the minor polypeptides was Sll1414. In addition, one or both of the Psb28 and Sll1130 proteins were removed by the $CaCl_2$ wash. However, only a small amount of the Sll1638 protein appeared to be removed by either $CaCl_2$ or Tris washing. It is possible that this cyanobacterial protein is more hydrophobic than PsbQ in plants and is not readily released from PSII. It is also noteworthy that most of the other newly identified proteins were not released during these treatments, consistent with the notion that they are components of PSII, and did not merely copurify with it.

These data also indicated that the isolated PSII complex is highly pure. None of the identified proteins is a known component of any of the other cyanobacterial membrane proteins, such as the photosystem I (PSI), cyt b_6f, and ATP synthase complexes. In addition, western immunoblot analysis failed to detect PsaA and PsaB, the reaction center proteins of PSI, in this PSII preparation (data not shown).

A number of earlier studies have documented a dimeric form of the PSII complex (e.g., *36*, *40*). The purified PSII complex from *Synechocystis* 6803 showed a single major peak at around 460 kDa on a size-exclusion chromatogram (data not shown), suggesting that the isolated PSII complexes from the HT-3 strain are present as dimers. It is noteworthy that similar analyses of purified PSII complexes from *Synechococcus elongatus* have estimated the mass of dimeric PSII as 480 kDa (*41*) and that from *Synechococcus vulcanus* as 580 kDa (*42*).

Spectroscopic Analysis. Fluorescence emission spectroscopy at 77 K is a sensitive mean to assess the presence of PSI and phycobilins in cyanobacterial subcellular preparations. When detergent-solubilized thylakoid membranes were excited at 600 nm (absorbed mainly by phycobilins) (Figure 3A), a high level of fluorescence was detected at around 650 nm, consistent with the presence of phycobiliproteins in such a sample (Figure 1). In comparison, such a fluorescence peak was not detected from the purified PSII complex, again indicating the absence of phycobilins from this preparation. Excitation of the membrane sample at 420 nm (absorbed mainly by Chl) resulted in a high emission peak at 720 nm from PSI and smaller peaks at 683.5 and 694 nm, both from PSII (Figure 3B). In contrast, the emission peaks from the PSII preparation were at 684 and 693 nm, and not at 720 nm, demonstrating the absence of PSI in this sample. The small hill at around 755 nm in the spectrum for the PSII sample was a satellite peak from Chls of PSII.

P700 is the reaction center Chl in PSI. The Chl/P700 ratio in the membranes from *Synechocystis* 6803 was 165, whereas it was $\geq$3300 in the purified PSII sample. Taking the immunoblot, low-temperature emission spectra and Chl/P700 data together, it is evident that there was no detectable PSI contamination in this PSII preparation.

Figure 4 displays dithionite reduced minus ferricyanide oxidized difference spectra of the isolated His-tagged PSII preparation. The spectrum from the untreated sample (Figure 4, spectrum A) had contributions from the α bands of two cytochromes, cyt b_{559} and cyt c_{550}. As shown in Figure 2, treatment with 1 M $CaCl_2$ released cyt c_{550} from this PSII preparation, and the spectrum from such a sample (Figure 4, spectrum B) showed a nearly symmetrical Gaussian peak (with a 10.2 nm width at half-maximum) centered around 560 nm that originated from cyt b_{559}. The Chl/cyt b_{559} ratio in this sample was $\sim$21 (Table 3). It is noteworthy that

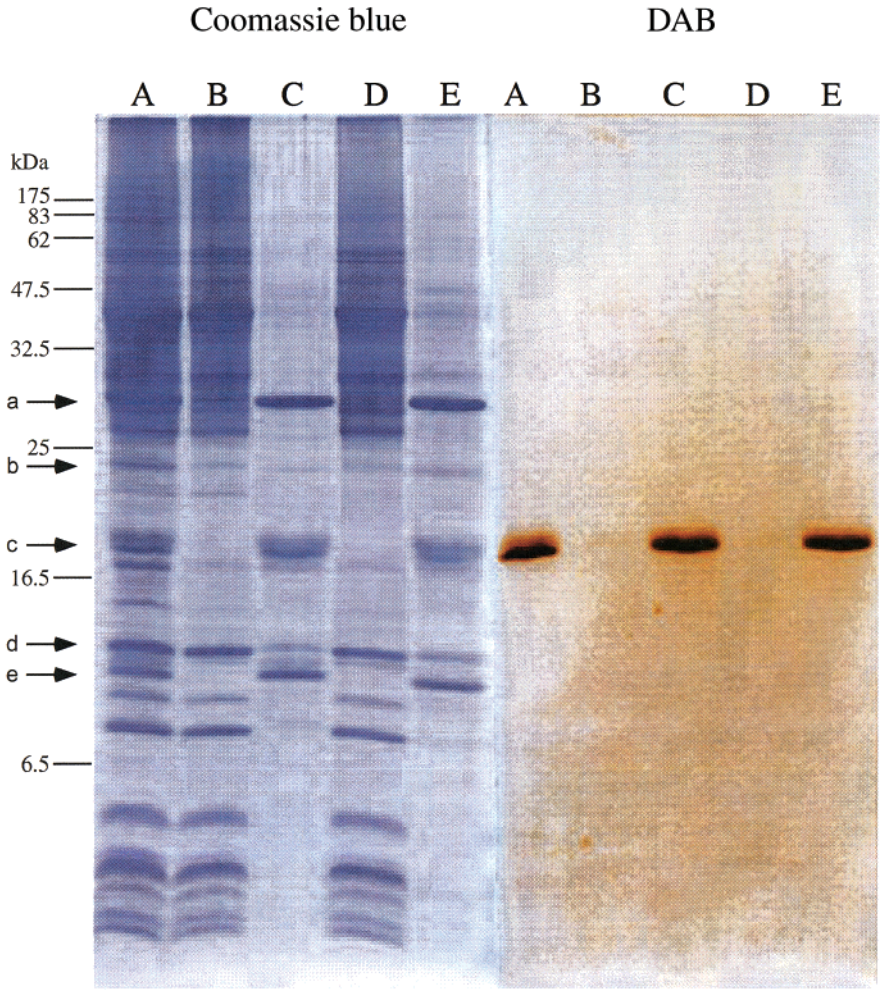

FIGURE 2: Treatment of purified PSII complexes with calcium chloride and Tris. The results of Coomassie blue staining and DAB staining are shown. A, purified PSII complex; B, $CaCl_2$-treated PSII complex; C, supernatant of $CaCl_2$-treated PSII complex; D, Tris-treated PSII complex; E, supernatant of Tris-treated PSII complex. The positions of molecular-weight standards are shown on the left. Samples equivalent to 10 μg Chl were applied to each lane of SDS−PAGE. Arrows on the left (a−e) denote principal polypeptides that were removed by $CaCl_2$ and Tris treatments.

Table 3: Cofactor Composition of Purified PSII Complex[a]

Chl/Q_A	Pheo/Q_A	β-carotene/Q_A	zeaxanthin/Q_A	Chl/Cyt b_{559}	Cyt b_{559}/Q_A	Ca/Q_A	Fe/Q_A	Mn/Q_A
41	2.4	11	1.3	21.6	1.9[b]	0.99	5.2	3.8
(2.0)	(0.20)	(0.12)	(0.10)	(2.7)	(0.23)	(0.11)	(0.39)	(0.09)

[a] Standard deviations are shown in parenthesis ($n = 3-10$). [b] In $CaCl_2$-treated PSII complex (see Figure 4).

majority of the cyt b_{559} in this His-tagged PSII preparation was in its low potential form (data not shown).

Composition of Cofactors. On the basis of the concentration of Q_A in this purified preparation, the number of Chl molecules per PSII reaction center was determined to be 41 (Table 3). The concentrations of other components were expressed based on this value. Pigment analysis using HPLC indicated that the number of pheophytin (Pheo) molecules per PSII center was 2.4. We suspect that some Chl molecules might have been converted to Pheo molecules during the extraction procedure. Eleven β-carotenes and ~1 zeaxanthin were also present per PSII complex. The number of cyt b_{559} hemes in PSII was 1.9 (see above). Atomic absorption spectroscopy revealed that the number of Ca, Fe, and Mn atoms in such a PSII complex were approximately 1, 5, and 4, respectively.

Oxygen Evolution Activities. In the presence of 0.5 mM DCBQ as an electron acceptor, the purified PSII complexes evolved oxygen at a rate as high as 2900 μmoles O_2 mg Chl^{-1} h^{-1} at 30 °C (Table 4). After incubation at 4 °C for 16 days in the dark, such a preparation retained 80% of the original activity, indicating that the purified PSII complexes were highly stable (data not shown). Addition of DCMU inhibited this activity nearly 90%. In the presence of duroquinone, an electron acceptor only from the Q_B-plastoquinone (*43*), the oxygen-evolving activity was 45% of that with DCBQ. DCMU inhibited this latter activity completely.

DISCUSSION

The recently described X-ray crystal structure of cyanobacterial PSII at a 3.8 Å resolution (*1*) has generated significant excitement in the study of the form and function of this large membrane protein complex. In addition, two high-resolution structures of cyt c_{550} have become available during recent months (*44*, *45*). As documented in this

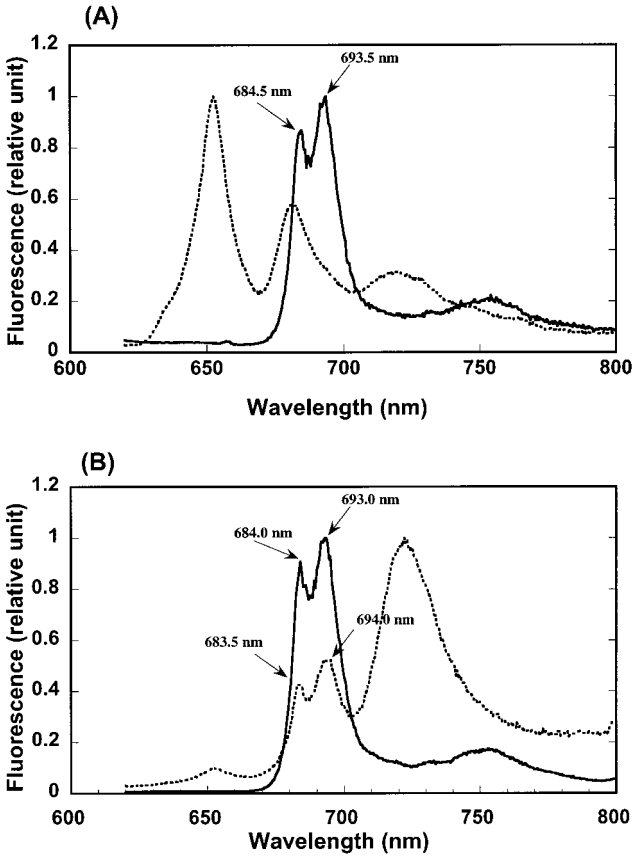

FIGURE 3: Fluorescence emission spectra of detergent solubilized membrane and purified PSII complex at 77 K. A, excitation at 600 nm; B, excitation at 420 nm. Solid line, purified PSII complex; dotted line, membrane. The samples were adjusted to 5 μg Chl mL^{-1}.

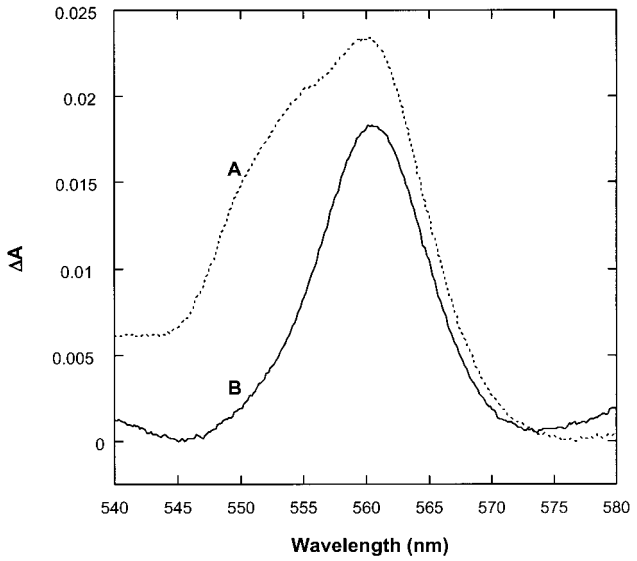

FIGURE 4: Difference (dithionite minus ferricyanide) absorbance spectra of purified PSII complex. A, PSII complex; B, $CaCl_2$-treated PSII complex. Each sample was adjusted to 20 μg Chl mL^{-1}, oxidized with potassium ferricyanide, and then reduced with sodium dithionite. See Experimental Procedures for further details.

manuscript, PSII has many polypeptide components, a number of which could not be identified in the available structure of PSII because of its intermediate resolution. For example, many of the small (<8 kDa) polypeptides have not been localized in the available structure.

Table 4: Oxygen-Evolving Activities of Purified PSII Complex[a]

	$CaCl_2$ 5 mM	DCMU 20 μM	activity (μmol O_2 mg Chl^{-1} h^{-1})	DCMU inhibition (%)
0.5 mM DCBQ	+	−	2910 (100)	−
0.5 mM DCBQ	+	+	286	90
1 mM FeCN	+	−	2100 (72)	−
1 mM FeCN	+	+	210	90
0.5 mM DCBQ + 1 mM FeCN	+	−	2610 (90)	−
0.5 mM DCBQ + 1 mM FeCN	−	−	1840 (63)	
0.5 mM DCBQ + 1 mM FeCN	+	+	513	80
1 mM duroquinone	+	−	1320 (45)	−
1 mM duroquinone	+	+	0	100

[a] DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FeCN, potassium ferricyanide.

In this work, we have used a genetically modified strain of *Synechocystis* 6803, in which a poly-histidine tag has been added at the carboxyl end of the CP47 protein (*9*) and purified the PSII complex using a gentle isolation procedure. The isolated PSII complex had 41 Chl molecules (Table 3), 9 more than that reported by Zouni et al. (*1*). During the preparation of this manuscript, Brudvig, Diner, and their colleagues reported on their studies on a similar His-tagged PSII preparation from *Synechocystis* 6803 (*11*). Similar to our finding, the number of Chl in their PSII preparation is 42. Our analysis also indicated the presence of one zeaxanthin molecule per PSII. Because cyanobacteria do not possess a xanthophyll cycle (*46*), the function of this pigment in cyanobacterial PSII is unknown. Eleven β-carotene molecules were found in this purified complex. According to Nanba and Satoh (*47*), only one such molecule is present in the D1/D2/cyt b_{559} "reaction center" preparation of spinach PSII. It is thus possible that 10 β-carotenes are shared between the large pigment-binding proteins CP43 and CP47. Tang and Diner have also determined the presence of 9.5 β-carotenes in their purified oxygen-evolving PSII core complex from *Synechocystis* 6803 (*48*). It is noteworthy that a recent investigation has shown that there is an even larger number (~17) of carotenoids in the same PSII preparation (*49*). About 14 of them are β-carotenes.

The number of cyt b_{559} hemes in our PSII preparation was ~1.9. This is in sharp contrast to the number of 1.1 cyt b_{559} hemes found by Lakshmi et al. in a similar His-tagged PSII preparation (*11*). The reasons behind such significantly different numbers are currently unknown, in particular, since the molar extinction coefficients of cyt b_{559} used in both of these studies are identical (*30*). An important modification in the purification of our PSII preparation is the use of histidine, instead of imidazole, during the elution of the protein complex from the metal affinity column (see Experimental Procedures). Interestingly, Crofts and colleagues also preferred the use of histidine over imidazole during the purification of a His-tagged cyt bc_1 complex from purple photosynthetic bacteria (*18*). In addition, we have biochemically eliminated cyt c_{550} to obtain a pure cyt b_{559} spectrum (Figure 4B). In contrast, Lakshmi and colleagues used a computational deconvolution method to derive a spectrum of cyt b_{559} (*11*). In the crystallized cyanobacterial PSII, one cyt b_{559} heme is present (*1*). However, it is noteworthy that Zouni et al. have commented that the "controversial issue of one or two cytochrome b_{559} per reaction center present in PSII. . . [remains]. . . unresolved, as this may depend on the

preparation and/or organism" (*1*). Hence, it is possible that one cyt b_{559} heme was lost during the purification of PSII complex from *Synechococcus elongatus* (*1*). In any case, further studies are necessary to resolve this apparent controversy in the number of cyt b_{559} hemes in a PSII complex.

Using such highly active preparations, we have identified many polypeptide components of PSII. It is noteworthy that a number of these proteins had blocked N-termini and could only be identified using mass spectroscopic analysis. Zheleva and co-workers used mass spectroscopic techniques to identify eight small proteins in a resolved PSII preparation from spinach (*50*). However, their SDS−PAGE system had "poor resolution. . . for proteins below 15 kDa", and they needed to use reverse phase HPLC to fractionate small molecular mass proteins. Recently, Szabo and co-workers have conducted mass spectroscopic analysis of proteins in cyanobacterial and plant PSII (*51*). Using MALDI analysis, they "tentatively" identified eight small PSII subunits in cyanobacteria. We have found that traditional N-terminal sequencing is a superior procedure to definitively identify such small proteins. Our analysis showed that the purified PSII complexes contained 12 polypeptides with apparent molecular weights below 8 kDa (Table 2). Although the *psbY* gene is present in the genome of *Synechocystis* 6803 (*52*, *53*), the presence of the PsbY polypeptide in purified cyanobacterial PSII complexes was confirmed for the first time during the current study. The *psbJ* gene is a member of the *psbEFLJ* gene cluster (*54*). Lind et al. have earlier shown the presence of this gene product in membranes from *Synechocystis* 6803 (*55*). However, the presence of PsbJ in cyanobacterial PSII was demonstrated for the first time using this His-tagged PSII preparation (*56*). The PsbZ protein, recently described in plant and algal PSII (*36*), and the small $PsbT_c$ protein, also involved in PSII dimerization in *Chlamydomonas* (*57*), were also identified in our cyanobacterial PSII preparation.

Thirty-one distinct proteins were identified in this isolated PSII complex. Moreover, six of the detected proteins remained unidentified during our analysis. Taken together, the number of known PSII components in plants and cyanobacteria exceeds 26. Hence, we have suggested a modified nomenclature in which proteins named after PsbZ are called Psb27, Psb28, etc. In consultation with Prof. M. Ikeuchi (*58*), we have renamed the Sll1398 protein as Psb28 and the Slr1645 protein as Psb27.

The data presented in this manuscript indicate that the isolated PSII preparation is free of contamination by PSI, phycobiliproteins, or any of the other major protein complexes in the thylakoid membrane. Two different forms of the FtsH protease were also present in the isolated PSII preparation (Table 2). Although a general protease for many cellular proteins, FtsH has recently been identified as one of the membrane bound proteases involved in the light-induced degradation of the D1 protein of PSII (*37*). Three of the minor proteins, the products of *lysS*, *gltA*, and *guaB* genes, have known functions unrelated to that of PSII (Table 2), and are probably "contaminations" in this PSII preparation. As shown in Table 2, five novel polypeptides are also present in this PSII preparation. Among them, the *sll1638* gene product is an unexpected finding. Sll1638 is a homologue of PsbQ, and according to the currently accepted model, PsbQ is absent from cyanobacterial PSII (*5*). The other four novel proteins have some predicted hydrophobic regions so that it is possible that they may copurify with PSII in detergent micelles. Preliminary analysis of knock out mutants in two of these five genes has suggested that they are involved in regulating PSII function in *Synechocystis* 6803 (Ohkawa, H., Chandler, L. E., Kashino, Y., Roose, J., Keren, N. and Pakrasi, H. B. Unpublished observations). However, more detailed biochemical analysis of such mutants is required before any of these five proteins is assigned a "Psb" designation.

In summary, we have identified all of the known proteins of cyanobacterial PSII in an isolated, highly active, PSII preparation from *Synechocystis* 6803. In addition, a number of novel proteins, including a homologue of the PsbQ protein, were found in this preparation. Genetic and biochemical studies are currently underway to examine the roles of these newly identified proteins in the structure, function, and biogenesis of PSII.

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