

## Evidence That the PsbK Polypeptide Is Associated with the Photosystem II Core Antenna Complex CP43\*

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**PsbK is encoded by the chloroplast *psbK* gene and is one of the small polypeptides of photosystem II (PSII). This polypeptide is required for accumulation of the PSII complex. In the present study, we generated an antibody against recombinant mature PsbK of *Chlamydomonas* and used it in Western blots to localize PsbK in the PSII core complex. PsbK was found in the thylakoid membranes, and purification of the PSII core complex from detergent-solubilized thylakoid membranes showed that PsbK is tightly associated with the PSII core complex. We used potassium thiocyanate to separate PSII into subcore complexes, including the D1/D2/cytochrome *b*<sub>559</sub> reaction center complex, CP47, and CP43, and we found that PsbK co-purifies with one of the core antenna complexes, CP43, during ion exchange chromatography. Subsequent gel filtration chromatography of the purified CP43 confirmed that PsbK is tightly associated with CP43. Steady-state levels of PsbK were also determined in *Chlamydomonas* mutants expressing various levels of PSII. Quantitative Western blotting revealed that the levels of PsbK in these mutants are approximately equal to those of CP43, suggesting that PsbK is stable only when associated with CP43 in the chloroplast. Together, our results indicate that PsbK is an integral part of the PSII complex and may participate in the assembly and stability of the PSII complex.**

Photosystem II (PSII)<sup>1</sup> catalyzes the light-induced transfer of electrons from water to plastoquinone (1). It is a large complex consisting of more than 20 polypeptides and a number of cofactors, including chlorophyll *a*, pheophytin *a*,  $\beta$ -carotene, plastoquinone, non-heme iron, and manganese atoms. The reaction center (RC), in which the photochemical reaction occurs, consists of two homologous polypeptides, D1 and D2, encoded by the *psbA* and *psbD* genes, respectively. The D1/D2 heterodimer contains all of the redox components involved in photochemical reactions. Cytochrome *b*<sub>559</sub> is composed of  $\alpha$ - and  $\beta$ -subunits encoded by the *psbE* and *psbF* genes, respectively, but is not directly involved in the main electron transfer of PSII. The two core antenna complexes, CP43 and CP47, are encoded by the *psbC* and *psbB* genes, respectively, and are

intimately associated with the reaction center. In addition to these major cofactor-binding subunits, the PSII complex contains extrinsic lumenal polypeptides, which are located in proximity to the O<sub>2</sub>-evolving manganese cluster and which optimize water-splitting activity.

Application of improved SDS-PAGE systems that can resolve small polypeptides together with microsequencing of the N-terminal amino acids has allowed identification of a number of small (<10 kDa) polypeptides in various PSII preparations (2, 3). Based on their deduced amino acid sequences, most of these small polypeptides contain a single transmembrane helix. These biochemical analyses have revealed not only the presence of the small polypeptides but also their approximate localization in PSII complex. PsbI, PsbT, PsbW, and cytochrome *b*<sub>559</sub> appear to be bound to RC because they are detected in preparations of the D1/D2 heterodimer (RC complex) (4–7). Other small polypeptides, including PsbH, PsbL, PsbM, and PsbX, are not detected in the RC complex but are found in the PSII core complex, which consists of RC and two core antenna complexes (7–9). This suggests that these small polypeptides are only weakly bound to the RC complex or that they are directly associated with CP43 and CP47.

PsbK is a small hydrophobic polypeptide of PSII with an apparent molecular mass of ~4 kDa. This polypeptide is encoded by the chloroplast *psbK* gene, is synthesized as a precursor with N-terminal extension, and is processed to a mature form (37 amino acid residues) (10). How tightly PsbK is bound to PSII complex is controversial. PsbK was first found in the O<sub>2</sub>-evolving PSII membrane fragment from spinach (10) but was not detected in the O<sub>2</sub>-evolving PSII core complex (11). These observations indicate that PsbK is loosely bound to the PSII complex and is easily released during solubilization and purification procedures. In contrast, PsbK was detected in the PSII core complex from cyanobacteria (12) and the green alga *Chlamydomonas reinhardtii* (8), indicating tighter association with the PSII complex.

Functional roles of many small PSII polypeptides remain to be elucidated. Disruption of the *psbE* and *psbF* genes eliminated accumulation of PSII complex, indicating that they are essential for assembly and/or stability of PSII complex (13). In addition, some of the small polypeptides (PsbH, PsbI, and PsbL) are required for optimal assembly and/or stability of PSII complex because disruption of their genes significantly reduced the level of the PSII complex (14–17). PsbT, another small PSII polypeptide, has a unique function in the repair of photodamaged PSII (5). Disruption of the *psbK* gene in cyanobacterium, *Synechocystis* PCC6803, reduces the steady-state level of PSII to a half of the wild-type level but allows photosynthetic cell growth (18). In contrast to the cyanobacterial mutants, mutants of the green alga, *C. reinhardtii*, were not able to grow photosynthetically and possessed a reduced level of PSII (19). Collectively, these studies indicate that PsbK plays an impor-

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<sup>1</sup> The abbreviations used are: PSII, photosystem II; RC, reaction center; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DM, *n*-dodecyl- $\beta$ -D-maltoside.

tant role in the stability and/or assembly of PSII complex.

Recent progress in the determination of the three-dimensional structure of the PSII complex reveals the presence of 22 transmembrane helices in the D1/D2 heterodimer and the two core antenna complexes as well as two transmembrane helices in cytochrome *b*<sub>559</sub>. In addition to these helices found in the major PSII polypeptides, 14 transmembrane helices have been detected in the PSII complex (20). These additional helices appear to be contributed by the small hydrophobic polypeptides associated with the PSII complex. However, the presence of the individual helices in the small hydrophobic polypeptides has not clearly been demonstrated because these polypeptides have neither cofactors nor structural characteristics that allow them to be identified.

In the present study, we raised an antibody against mature *Chlamydomonas* PsbK and used it to localize PsbK in the PSII core complex. In addition, we developed procedures to purify the PSII core and subcore complexes. Finally, we took advantage of a collection of *Chlamydomonas* PSII-deficient mutants to study the roles of the PSII polypeptides in PsbK stability. We found that PsbK is tightly bound to CP43 and that CP43 stabilizes PsbK *in vivo*.

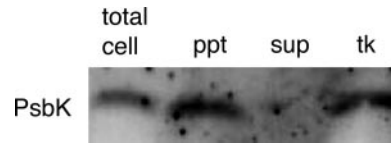
#### EXPERIMENTAL PROCEDURES

**Strains and Growth Condition**—In these studies, we utilized the *C. reinhardtii* wild-type strain 137C, the PSII-deficient mutants, Fud7, nac.2.26, 222E, F34, and Fud44, and the *psbK*-deficient mutant ( $\Delta$ *psbK*). Cells were grown to mid-log phase ( $2\text{--}5 \times 10^6$  cells ml<sup>-1</sup>) in Tris acetate-phosphate medium at 25 °C (21).

**Overexpression of Mature PsbK**—The region encoding mature PsbK was amplified by PCR using a pair of synthetic oligonucleotides, 5'-CATATGAACTTCCTGAAGCATAC-3' and 5'-CATATGTTAACG-GAACTAACAGCTGC-3' (22). *Nde*I sites were introduced at both ends of the amplified DNA fragment. The amplified fragment was digested with *Nde*I and was cloned in the pET3X-c vector at the *Nde*I site in the right orientation. Expression was induced in *Escherichia coli* with 1 mM isopropylthio- $\beta$ -D-galactoside. The recombinant protein was purified by SDS-PAGE according to Ref. 23, eluted electrophoretically from the gel, and injected into rabbits.

**Western Blotting**—Polypeptides from cells or thylakoid membranes were solubilized with 2% SDS and 0.1 M dithiothreitol at 100 °C for 1 min, whereas polypeptides in PSII preparations were solubilized at room temperature for 60 min. Except for PsbK, the polypeptides were separated by urea-SDS-PAGE as described in Ref. 24. To improve the isolation of small polypeptides, PsbK was separated by SDS-PAGE according to Ref. 23. Separated polypeptides were electrophoretically transferred to nitrocellulose filters and then probed with specific polyclonal antibodies. The signals were visualized by enhanced chemiluminescence. A dilution series of total thylakoid polypeptides was used as a calibration standard to estimate the amount of PSII polypeptides in thylakoid membranes. Antibodies raised against D1 and PsaA were kindly provided by Drs. M. Ikeuchi (University of Tokyo) and K. Redding (University of Alabama), respectively.

**Purification and Dissociation of PSII Complexes**—Thylakoid membranes were isolated and purified by discontinuous sucrose density gradient centrifugation as described in Ref. 25. The thylakoid membranes (0.8 mg of chlorophyll ml<sup>-1</sup>) were solubilized with 0.8% (w/v) *n*-dodecyl- $\beta$ -D-maltoside (DM), and the resulting extracts were fractionated by sucrose density gradient centrifugation as described previously (26). The sucrose gradient contained a linear concentration of sucrose from 0.1 to 1.3 M, 5 mM Tricine-NaOH, pH 8.0, and 0.05% DM. Four green bands were separated after centrifugation at  $141,000 \times g$  (SW28, Beckman) at 4 °C for 24 h. The PSII core complex was purified from the PSII-enriched fraction by ion-exchange chromatography with DEAE-Toyopearl 650S (Tosoh, Tokyo, Japan). Fractions were eluted with a linear gradient of NaCl (25–175 mM) in 50 mM Tris-HCl, pH 8.0, and 0.05% DM. A dilution series of total thylakoid polypeptides was used as a calibration standard. The PSII subcore complexes were dissociated from the PSII core complex by incubation with 0.75 M KSCN and 0.05% DM in the dark at 30 °C for 1 h. Dissociation was terminated by a 90-fold dilution with cold 0.05% DM. The resulting subcore complexes were loaded onto a DEAE column equilibrated with 50 mM Tris-HCl, pH 8.0, and 0.05% DM, and fractions were eluted with a linear gradient of



**FIG. 1. Localization of PsbK in cells.** *Chlamydomonas* wild-type cells were broken with a French pressure cell, and the resulting cell extracts were fractionated into pellet (*ppt*) and supernatant (*sup*) by centrifugation. Thylakoid membranes (*tk*) were purified by discontinuous sucrose density gradient centrifugation from the pellet. PsbK was separated by SDS-PAGE, blotted onto nitrocellulose filter, and probed with an antibody against PsbK.

NaCl (0–200 mM) in 50 mM Tris-HCl, pH 8.0, and 0.05% DM. Gel filtration chromatography of subcore complexes was performed using a HiLoad 16/60 Superdex 200pg column (Amersham Biosciences) and fractions were eluted with 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.05% DM, at a flow rate of 1 ml min<sup>-1</sup>.

#### RESULTS

**PsbK Is Associated Exclusively with PSII Core Complex**—To localize PsbK in *Chlamydomonas* cells, we generated an antibody against the mature form of PsbK. Wild-type *Chlamydomonas* cells were broken with a French pressure cell, and the resulting cell extracts were separated into soluble and insoluble portions by centrifugation. Western blotting revealed that PsbK is present in the pellet, whereas PsbK is not detected in the supernatant (Fig. 1). This indicates that PsbK is located in membrane fraction, consistent with the fact that PsbK is a hydrophobic polypeptide with a putative transmembrane helix. We further found that PsbK is present in thylakoid membranes purified from the pellet by discontinuous sucrose density gradient centrifugation. Although the purified thylakoid membranes may be contaminated by submitochondrial membranes (27), it is very unlikely that the chloroplast-encoded PsbK is translocated from chloroplast into mitochondria. Thus, these results indicate that PsbK is located exclusively in the thylakoid membranes.

It has already been reported that the *Chlamydomonas* PSII core complex contains PsbK (8). However, it is not clear whether all of the PsbK in the thylakoid membranes remains associated with the purified PSII core complex or whether part of the PsbK is released during thylakoid membrane solubilization or purification of the extracts. To examine these possibilities, the thylakoid membranes were solubilized with DM and fractionated by sucrose density gradient centrifugation. As shown in Fig. 2A, four green bands were present in the density gradient and were designated as A-1, A-2, A-3, and A-4, starting from the top of the gradient. The polypeptide composition of the fractions was analyzed by SDS-PAGE as shown in Fig. 2B. Band A-1 (fractions 7–11) contained two major proteins of ~25–30 kDa and therefore corresponds to light-harvesting complex of photosystem II (LHCII). Bands A-3 (fractions 19–21) and A-4 (fractions 24–27) contained PSI polypeptides and therefore correspond to PSI complexes. The PSII core complex was enriched in band A-2. This band also contained PSI polypeptides but fewer than band A-3 (Fig. 2C). Western blotting clearly indicated that PSII polypeptides as well as PsbK are found only in band A-2 (Fig. 2C). Dissociation of PsbK from the PSII core complex was not observed during solubilization and purification, indicating that PsbK is tightly associated with the PSII core complex.

**Co-purification of PsbK and CP43**—Because band A-2 contained not only the PSII complex but also the PSI complex, we fractionated this band by ion-exchange chromatography on DEAE (Fig. 3). The elution profile shows the separation of three peaks (*B-1b*, *B-2*, and *B-3*) and two shoulders (*B-1a* and *B-4*). The profile of Coomassie Brilliant Blue-stained polypeptides

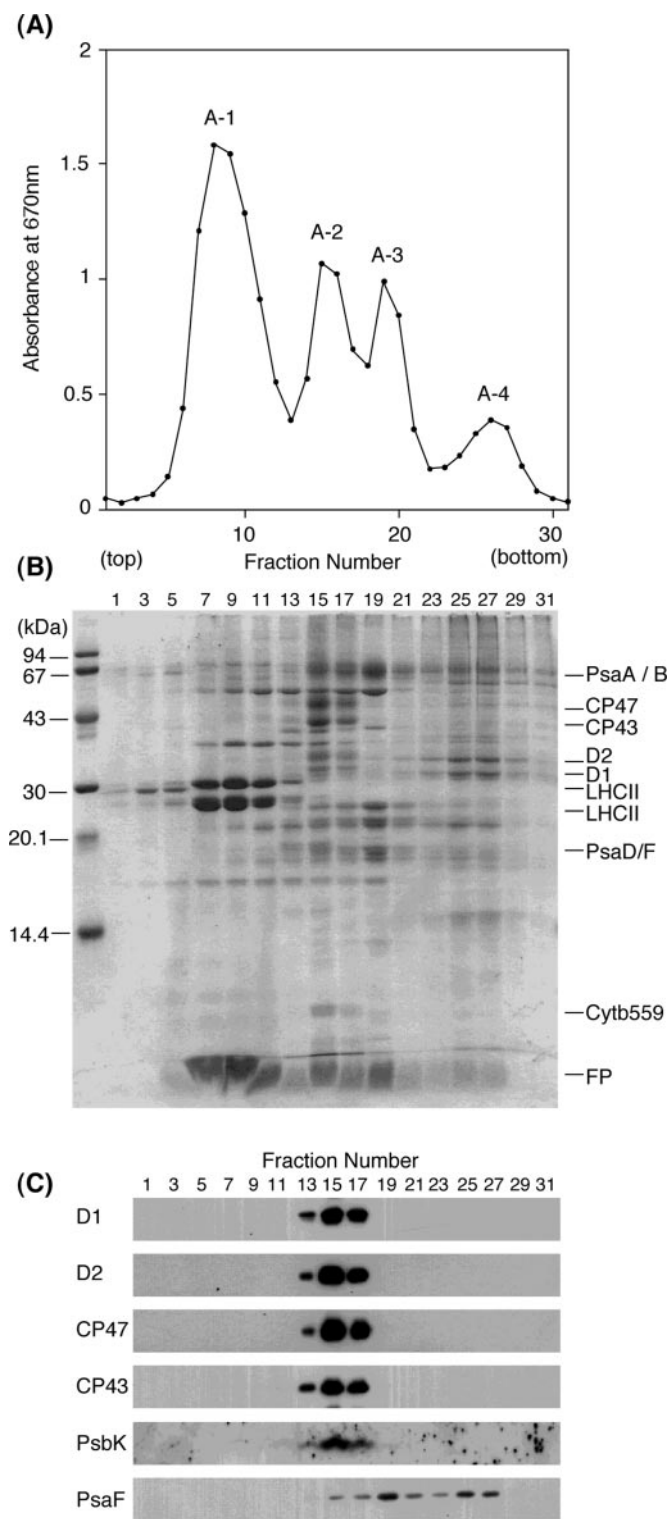


FIG. 2. Fractionation of chlorophyll-protein complexes by sucrose density gradient centrifugation. Purified thylakoid membranes were solubilized with DM, and the resulting extracts were separated by linear sucrose density gradient centrifugation. The resulting gradient was fractionated from the top to the bottom, and the elution was monitored by absorption at 670 nm (A). Polypeptides in the fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (B). FP represents free pigments. PSII and PSI polypeptides were detected by Western blotting using antibodies against D1, D2, CP47, CP43, PsbK, and PsaF (C).

(Fig. 3B) and Western blotting (Fig. 3C) indicated that *B-1b* and *B-2* contain PSI polypeptides, whereas *B-3* contains all the major PSII polypeptides. In addition, *B-4* contained major PSII

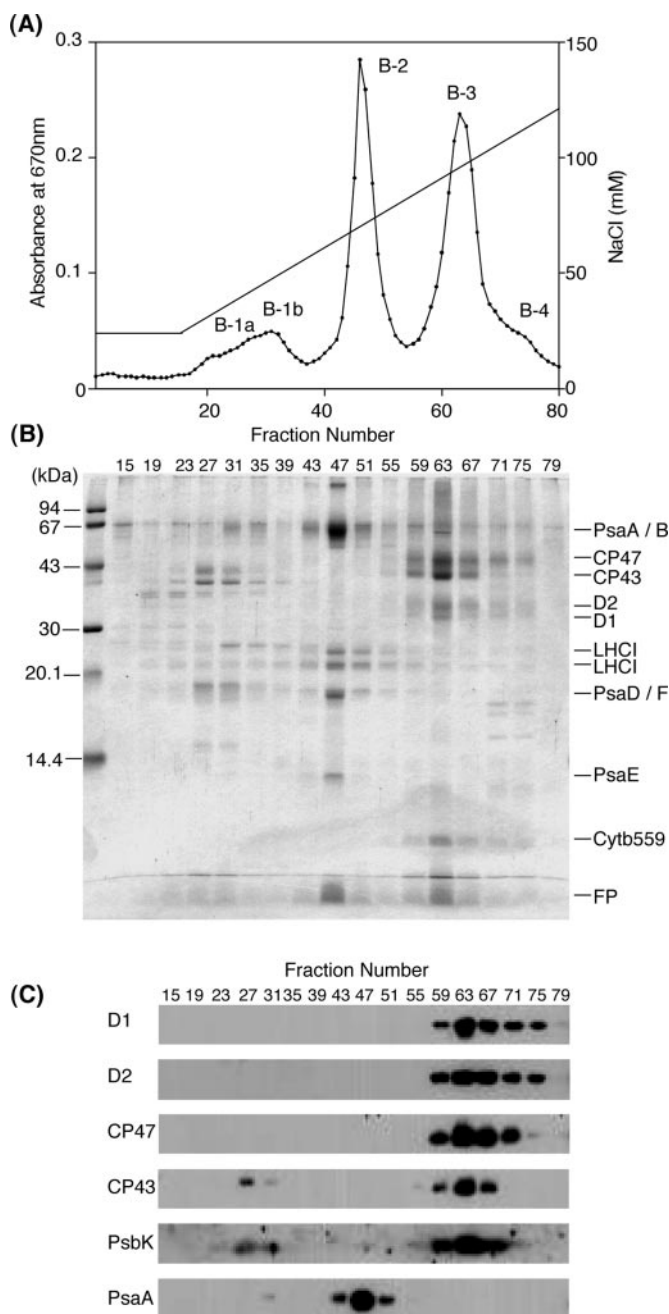


FIG. 3. Purification of the PSII core complex by ion-exchange chromatography on DEAE. Band A-2, obtained by sucrose density gradient centrifugation, was loaded onto a DEAE column. Chlorophyll-protein complexes were eluted with a gradient of NaCl (25–175 mM) in 50 mM Tris-HCl, pH 8.0, and 0.05% DM. The elution was monitored by absorption at 670 nm (A). Polypeptides in the fractions were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (B). FP represents free pigments. PSII polypeptides were detected by Western blotting using antibodies against D1, D2, CP47, CP43, and PsbK (C).

polypeptides except for CP43 (fractions 71 and 75) and thus represented CP43-depleted PSII core complex. A small amount of CP43 was released during purification and was separated into *B-1a* fractions. These results suggest that CP43 is associated with the PSII core complex more weakly than the other major PSII polypeptides. As expected, almost all of the PsbK was detected in *B-3*, confirming that PsbK is a component of the PSII core complex. However, PsbK was absent from *B-4*, whereas a small amount of PsbK was detected in *B-1a*. These observations strongly suggest that PsbK is bound to the dissociated CP43.



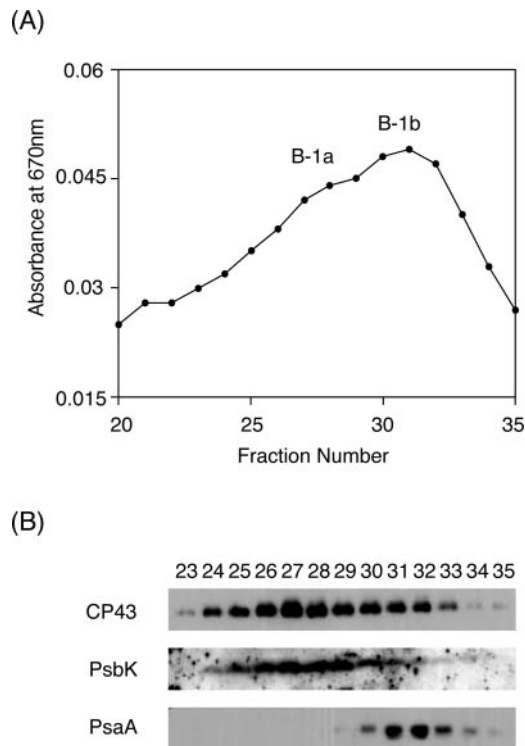


FIG. 4. The elution profile around *B-1a* and *B-1b*. The elution was monitored as described in the legend for Fig. 3 (A). CP43, PsbK, and PsaA in each fraction were detected by Western blotting (B).

To investigate the co-purification of PsbK and CP43 in more detail, we analyzed every fraction from 23 to 35 (Fig. 4). We found that the peak for CP43 occurred in fraction 27, whereas that for PsaA was in fraction 32. The elution profile for PsbK was very similar to that for CP43, supporting the idea that PsbK co-purifies with CP43.

**Association of PsbK with CP43**—Our observations suggest the novel finding that PsbK is localized in the PSII subcore complex. To reveal definitely the association of PsbK with CP43, we dissociated the purified PSII core complex into subcore complexes, without releasing free pigments, by using the chaotropic reagent KSCN (5, 28). After treatment of the PSII core complex with 0.75 M KSCN, the subcore complexes were fractionated by ion-exchange on DEAE. The elution profile included three peaks: *C-1*, *C-2*, and *C-3* (Fig. 5A). Western blotting using antibodies against major PSII polypeptides revealed that *C-1* corresponds to CP43, whereas *C-2* corresponds to CP47. *C-3* contains the PSII reaction center because it consisted of both D1 and D2 but lacked CP43 and CP47 (Fig. 5B). These findings demonstrate that the PSII core complex was completely dissociated into subcore complexes with KSCN. Furthermore, PsbK was only present in *C-1* and was not detected in *C-2* or *C-3*.

To confirm that PsbK is associated with CP43, we further separated *C-1* by gel filtration on Superdex-200pg (data not shown). Gel filtration resulted in a single peak containing CP43 and PsbK, indicating that the CP43 is homogeneous and that PsbK specifically and tightly binds to CP43. The apparent size of CP43 was estimated as 150–170 kDa, suggesting that the CP43 in *C-1* may be a dimer because the monomeric CP43 is calculated to be 70 kDa or may be a monomer bound by a significant amount of detergent.

**PsbK Is Stabilized by CP43**—We examined the correlation between PsbK and PSII levels by taking advantage of the large collection of photosynthetic mutants of *C. reinhardtii*. Deficiency of one of the PSII proteins can affect assembly and/or

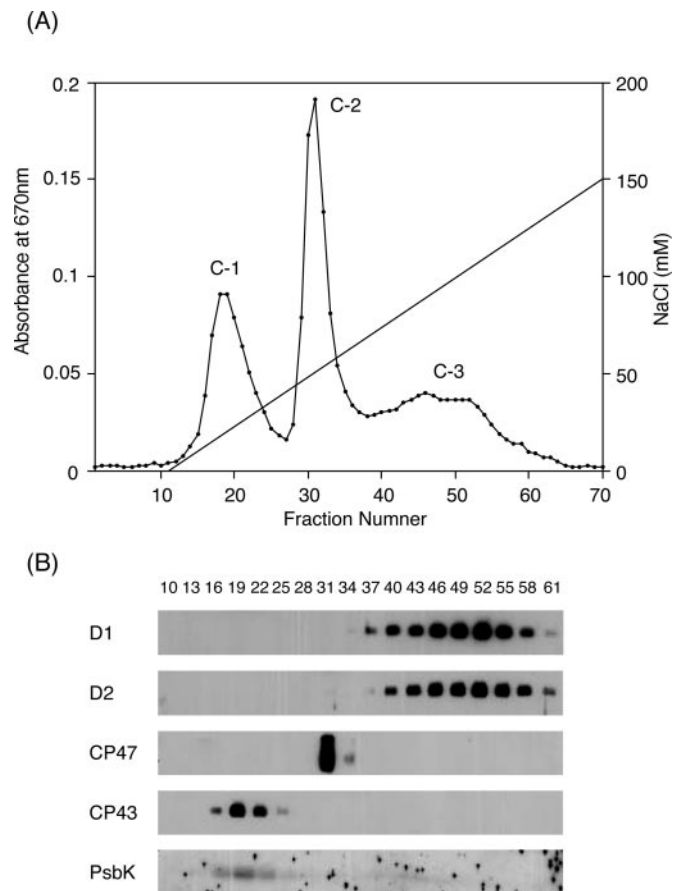


FIG. 5. Dissociation of the PSII core complex into subcore complexes. The purified PSII core complex (*B-3*) was dissociated into subcore complexes with 0.75 M KSCN at 30 °C for 1 h. The resulting subcore complexes were diluted with cold 0.05% DM solution and loaded onto the column. Subcore complexes were eluted with a gradient of NaCl (0–150 mM) in 50 mM Tris-HCl, pH 8.0, and 0.05% DM. The elution was monitored by absorption at 670 nm (A). PSII polypeptides were detected by Western blotting using antibodies against D1, D2, CP47, CP43, and PsbK (B).

stability of the whole PSII complex, leading to a decreased amount of the other PSII proteins (3). This pleiotropic effect is observed in the PSII mutants that lack D1 (Fud7) (29), D2 (nac.2.26) (30), CP43 (F34) (31), CP47 (222E) (32), or OEE1 (Fud44) (33) (Fig. 6). D1, D2, and CP47 were not detected in the mutants deficient for these polypeptides, including Fud7, nac.2.26, and 222E. However, CP43 was found at 28–36% of the wild-type level in these mutants (Table I). A similar phenotype was observed in Fud44, which lacks OEE1. In contrast to the other mutants, F34 is deficient in CP43 but still contains D1, D2, and CP47 at 12–16% of the wild-type levels.

Interestingly, the level of PsbK in Fud7, nac.2.26, 222E, and Fud44 was 24–43% of the wild-type level, whereas F34 did not contain PsbK. Apparently, the steady-state level of PsbK in the various PSII-deficient mutants was approximately equal to that of CP43. It appears that accumulation of PsbK is dependent on the presence of CP43, or vice versa. As reported previously (19), the level of PSII polypeptides in  $\Delta psbK$  mutants is ~10% of the wild-type level. In the present study, the amount of PSII in the  $\Delta psbK$  mutants was re-evaluated by Western blotting using an antibody against D1. We generated a dilution series of D1 by mixing wild-type cells with Fud7 mutant cells that lack D1, thereby maintaining the concentration of total cellular protein, and used it to create a calibration curve for D1 levels. We estimated that the level of D1 in  $\Delta psbK$  mutants is 15–18% of the wild-type level (Table I). We also found that the

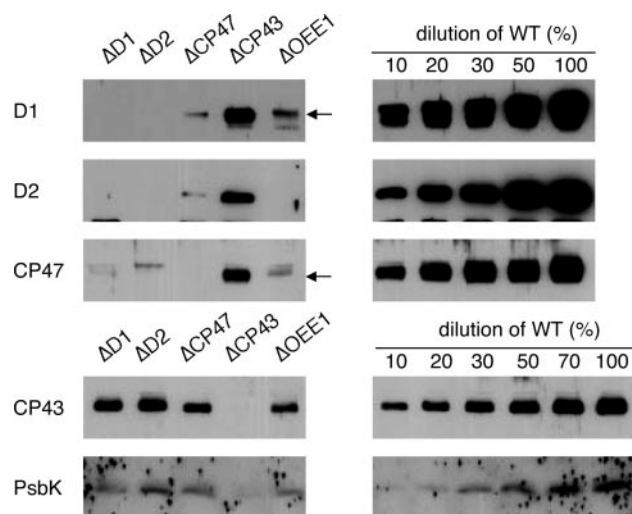


FIG. 6. **PSII polypeptides in the *Chlamydomonas* PSII mutants.** Steady-state levels of PSII polypeptides (D1, D2, CP47, CP43, and PsbK) were determined by Western blotting using a dilution series of wild type (WT) as a standard for calibration. Mutants  $\Delta$ D1,  $\Delta$ D2,  $\Delta$ CP47,  $\Delta$ CP43, and  $\Delta$ OEE1 correspond to Fud7 (29), nac.2.26 (30), 222E (31), F34 (32), and Fud44 (33) strains, respectively.

light-induced  $O_2$ -evolving activity, measured in the presence of the artificial electron acceptor phenyl-*p*-benzoquinone, was 10–20% of the wild-type level. Thus, the PSII complex that exists in the  $\Delta$ psbK mutant cells is functional. Although there is a significantly reduced level of PSII in  $\Delta$ psbK mutants, the steady-state level of the major PSII proteins, D1, D2, CP43, and CP47, is almost identical (Table I). Thus, it can be concluded that PsbK is stable only when associated with CP43.

To analyze whether the major PSII polypeptides are assembled into a stable complex in the  $\Delta$ psbK mutants, the thylakoid membranes were solubilized with DM and fractionated by sucrose density gradient centrifugation (Fig. 7). The separation pattern was similar to that of the wild type shown in Fig. 2 except that A-2 was reduced and thus separated as a shoulder of A-3. Because A-2 contained both PSI and PSII complexes, the significant reduction in A-2 resulted from the decreased amount of PSII complex. As expected, the major PSII polypeptides, D1, D2, CP47 and CP43, were detected in A-2 (fractions 17 and 19), whereas PSI polypeptide (PsaF) was found in A-2, A-3, and A-4. However, a small portion of CP43 was detected in A-1 (fractions 13 and 15). This suggests that, when PsbK is absent, some CP43 dissociates from the PSII core complex and separates into the upper region of the sucrose gradient due to reduced CP43 binding to the PSII core complex. Alternatively, a small amount of CP43 may remain unbound to the CP47-RC subcore complex *in vivo* because of reduced efficiency of CP43 assembly into the subcore complex. In support of the latter explanation, we found that there was no increase in CP43 dissociation when the thylakoid membranes were solubilized with higher concentrations (1.0–1.4%) of DM.

#### DISCUSSION

**PsbK Is a New Component of the Core Antenna Complex, CP43—PsbK** was first found in an  $O_2$ -evolving PSII membrane preparation isolated from spinach (10). How tightly PsbK binds to the PSII complex has been controversial. PsbK is lost during preparation of  $O_2$ -evolving PSII core complexes that lack light-harvesting complex of photosystem II (LHCII) but retain high  $O_2$ -evolving activity (12). This suggests that PsbK binds weakly to the PSII complex. However, PsbK has been detected in *Chlamydomonas* PSII core complexes that lack extrinsic luminal polypeptides and  $O_2$ -evolving activity (8). More recently, a

dimeric form of the CP47-RC subcore complex (CP43-deficient PSII core complex) from spinach was found to retain PsbK, suggesting a more tight binding of PsbK to PSII complex (34).

In the present study, we raised an antibody against mature recombinant PsbK and successfully used it in Western blots to localize PsbK in the PSII core complex. Our results clearly demonstrated that PsbK is present in the thylakoid membranes and co-purifies with the PSII core complex. In addition, we showed that all of the PsbK is specifically associated with CP43. This observation was further supported by the analyses of the various PSII-deficient mutants. Mutants deficient in D1, D2, CP47, or OEE1 contained some of both CP43 and PsbK, whereas the mutant lacking CP43 also lacked PsbK. These findings are novel because small polypeptides have not been reported previously in purified CP43 preparations. Also, transmembrane helices other than the six helices of CP43 were not found in electron microscopic analyses of spinach CP43 microcrystals (35).

The three-dimensional structure of the cyanobacterial PSII complex determined at 3.8-Å resolution reveals a number of small hydrophobic polypeptides that have a single transmembrane helix (20). Two polypeptides of cytochrome  $b_{559}$  have been localized because they bind the heme. However, it has been difficult to assign the other small polypeptides because they contain neither cofactors nor characteristic structures. Recently, an improved three-dimensional structure of the PSII complex has been determined at 3.7-Å resolution (36). According to this structure, one transmembrane helix with a bend is in close proximity to CP43. This helix was assigned PsbK because it is the only polypeptide that contains Pro in the hydrophobic region, which is expected to form the bend in the transmembrane helix. This observation agrees with our findings that PsbK co-purifies with CP43.

Although the monomeric form of the CP47-RC subcore complex from spinach has been reported to lack PsbK, the dimeric form contains PsbK (34). This finding is inconsistent with our observations that all of the PsbK co-purifies with CP43. According to the three-dimensional structure of cyanobacterial PSII complex, PsbK is present in the space formed between D2 and CP43 but is located much closer to CP43 than to D2 (20, 36). It is unlikely that PsbK is located at a different region in the spinach PSII complex, but it is possible that PsbK is positioned closer to D2 in spinach. If this is the case, the affinity of PsbK for RC could be higher than for CP43 so that PsbK might remain associated to the dimeric form of CP47-RC. However, this is not the case in *Chlamydomonas* because the CP47-RC subcore complex elutes in B-4, which lacks PsbK. Although it is not known whether CP47-RC is monomeric or dimeric, it is clear that the dissociated CP43 binds PsbK in *Chlamydomonas*.

In contrast to CP43, PSII-RC preparations consisting of D1/D2 heterodimer contain several small hydrophobic polypeptides, including the  $\alpha$ - and  $\beta$ -subunits of cytochrome  $b_{559}$  encoded by the chloroplast *psbE* and *psbF* genes (6), the chloroplast-encoded *psbI* gene product (9), and the nuclear-encoded *psbW* gene product (4). Recently, Western blotting detected the chloroplast-encoded PsbT in a PSII-RC preparation (5). Two-thirds of PsbT was present in the PSII-RC, whereas the other one-third was released from the PSII-RC. It is not yet clear how tightly PsbI and PsbW are associated with PSII-RC. The three-dimensional structure of the PSII complex suggests an intimate association of additional small transmembrane polypeptides with the D1/D2 heterodimer. Furthermore, several transmembrane helices are present in proximity to core antenna complexes, CP43 and CP47. More intensive characterization of polypeptide composition of the purified CP43 and CP47 preparations and a more detailed analysis of the crystal

TABLE I  
Levels of PSII polypeptides in *Chlamydomonas* PSII mutants

Values represent means  $\pm$  standard deviation ( $n = 3$ ).

Strains	Phenotype	% of wild-type level				
		D1	D2	CP47	CP43	PsbK
Fud7	$\Delta$ D1	0	0	0	31 $\pm$ 2	24 $\pm$ 3
nac.2.26	$\Delta$ D2	0	0	0	36 $\pm$ 4	43 $\pm$ 2
222E	$\Delta$ CP47	<<10	<<10	0	28 $\pm$ 7	31 $\pm$ 2
F34	$\Delta$ CP43	16 $\pm$ 1	13 $\pm$ 2	12 $\pm$ 2	0	0
Fud44	$\Delta$ OOE1	<<10	0	<<10	18 $\pm$ 3	24 $\pm$ 4
$\Delta$ psbK	$\Delta$ PsbK	16 $\pm$ 2	18 $\pm$ 1	15 $\pm$ 2	17 $\pm$ 2	0

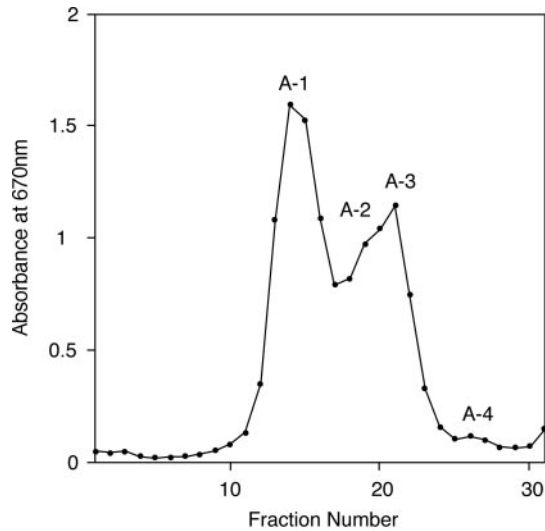


FIG. 7. Separation of chlorophyll-protein complexes from the  $\Delta$ psbK mutant. The thylakoid membranes of the *Chlamydomonas*  $\Delta$ psbK mutant were solubilized with DM and separated by sucrose density gradient centrifugation as described in the legend for Fig. 2. PSI and PSII polypeptides were analyzed by Western blotting using antibodies against D1, D2, CP47, CP43, and PsbK.

structure of the PSII complex will be required for full assignment of the small hydrophobic polypeptides in the PSII core complex.

**Involvement of PsbK in Assembly and Stability of PSII Complex**—Assembly of PSII complex is predicted to be a multistep process because a large number of subunits and cofactors must be sequentially integrated into a functional pigment protein complex (37). As described in the present studies and in Ref. 38, CP43 levels in Fud7, nac.2.26, 222E, and Fud44 mutants are 28–36% of wild-type levels, whereas D1, D2, and CP47 levels are 12–16% of wild type in the CP43-deficient mutant F34. Nearly stoichiometric levels of D1, D2, and CP47 occur in F34 mutant cells, suggesting that the CP47-RC subcore complex is assembled in the absence of CP43. This possibility is supported by CP47-RC complexes found in *psbC* deletion mutants of cyanobacteria (39). Our observation that F34, but not the other PSII mutants, lacks PsbK indicates that PsbK is stable when

CP43 is present, likely when it is bound to CP43. Therefore, it appears that, after synthesis, PsbK immediately binds to CP43 to form a PsbK-CP43 complex that is subsequently integrated into the CP47-RC subcore complex, thus forming the PSII core complex. If this is the case, assembly of PsbK-CP43 and CP47-RC subcore complexes may occur independently.

In the previous report (19), it was proposed that the absence of PsbK affects the assembly and/or stability of the whole PSII complex because the mutant cells of *Chlamydomonas* synthesized PSII polypeptides normally but contained only 10–20% of the wild-type level of PSII complex. The PSII complex was stable in the thylakoid membranes isolated from the  $\Delta$ psbK cells but was unstable *in vivo* (data not shown), suggesting that assembled PSII complex lacking PsbK is subject to proteolytic degradation. A structural modification of the PSII complex induced by the absence of PsbK could be recognized by protease(s) in the chloroplast. In addition to the instability of the PSII complex, the absence of PsbK might also affect the assembly of CP43 into the CP47-RC subcore complex. In fact, a small amount of dissociated CP43 was detected on the sucrose density gradient of thylakoid extracts from  $\Delta$ psbK. The amount of dissociated CP43 remained constant when the concentration of DM was increased to enhance thylakoid membrane solubilization, indicating that the free CP43 might be derived from unassembled CP43 rather than from partial dissociation from the core complex by detergent. These observations strongly suggest that PsbK located between RC and CP43 may facilitate assembly of CP43 into CP47-RC subcore complex. We also found that the absence of CP43 leads to a significantly reduced amount of PSII complex. Thus, the decreased steady-state level of PSII complex in  $\Delta$ psbK cells may be partly due to a defect in this assembly step. Collectively, these results suggest that PsbK affects both the assembly and the stability of the PSII complex.

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**Evidence That the PsbK Polypeptide Is Associated with the Photosystem II Core  
Antenna Complex CP43**

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