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Absence of the PsbQ Protein Results in Destabilization of the PsbV Protein and Decreased Oxygen Evolution Activity in Cyanobacterial Photosystem II*

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We have previously reported that cyanobacterial photosystem II (PS II) contains a protein homologous to PsbQ, the extrinsic 17-kDa protein found in higher plant and green algal PS II (Kashino, Y., Lauber, W. M., Carroll, J. A., Wang, Q., Whitmarsh, J., Satoh, K., and Pakrasi, H. B. (2002) *Biochemistry* 41, 8004–8012) and that it has regulatory role(s) on the water oxidation machinery (Thornton, L. E., Ohkawa, H., Roose, J. L., Kashino, Y., Keren, N., and Pakrasi, H. B. (2004) *Plant Cell* 16, 2164–2175). In this work, the localization and the function of PsbQ were assessed using the cyanobacterium *Synechocystis* sp. PCC 6803. From the predicted sequence, cyanobacterial PsbQ is expected to be a lipoprotein on the luminal side of the thylakoid membrane. Indeed, experiments in this work show that upon Triton X-114 fractionation of thylakoid membranes, PsbQ partitioned in the hydrophobic phase, and trypsin digestion revealed that PsbQ was highly exposed to the luminal space of thylakoid membranes. Detailed functional assays were conducted on the *psbQ* deletion mutant (Δ*psbQ*) to analyze its water oxidation machinery. PS II complexes purified from Δ*psbQ* mutant cells had impaired oxygen evolution activity and were remarkably sensitive to NH_2OH, which indicates destabilization of the water oxidation machinery. Additionally, the cytochrome c_{550} (PsbV) protein partially dissociated from purified Δ*psbQ* PS II complexes, suggesting that PsbQ contributes to the stability of PsbV in cyanobacterial PS II. Therefore, we conclude that the major function of PsbQ is to stabilize the PsbV protein, thereby contributing to the protection of the catalytic Mn_4-Ca_1-Cl_x cluster of the water oxidation machinery.

Photosystem II (PS II)[2] evolves molecular oxygen by oxidizing water molecules using the driving force of light energy (1).

This water-splitting reaction is catalyzed by an inorganic Mn_4-Ca_1-Cl_x cluster and assisted by several extrinsic proteins located on the luminal surface of PS II complex (2). The subunit composition of these extrinsic proteins differs between cyanobacterial PS II and that from higher plants and green algae. PS II of green algae and higher plants contains three luminal extrinsic proteins: PsbO (33-kDa manganese-stabilizing protein), PsbP (24-kDa protein), and PsbQ (17-kDa protein). In contrast, cyanobacterial PS II complexes contain the extrinsic proteins PsbO, PsbU (12-kDa protein), and PsbV (cytochrome c_{550}) (2–4), in which only PsbO is common to both systems. More recently, PsbP and PsbQ have been identified in cyanobacterial PS II (5, 6).

Biochemical removal and reconstitution experiments have aided in our understanding of the roles of the plant PS II extrinsic proteins. The PsbO protein stabilizes the manganese cluster and is crucial for PS II oxygen evolution activity, whereas PsbP and PsbQ are necessary for oxygen evolution at physiological Ca^{2+} and Cl^- concentrations (2, 7–10). Specifically, reconstitution of PsbP to PS II complexes containing PsbO reduces the Ca^{2+} requirement, while further reconstitution of PsbQ restored oxygen evolution activity at a moderate rate at low concentrations of Cl^- (9, 11). These removal/reconstitution experiments indicate that PsbP and PsbQ offer the binding sites of Ca^{2+} and Cl^- ions or stabilize these ions within the PS II complex (2, 7–9). Together the three extrinsic proteins, PsbO, PsbP, and PsbQ, form a barrier around the manganese cluster protecting the catalytic center from small reductants, such as NH_2OH (12).

The cyanobacterial extrinsic proteins PsbO, PsbU, and PsbV can be removed by high salt wash (*e.g.* by 1 M $CaCl_2$, but not by 1 M NaCl) (3) and reconstituted to PS II core complexes (4). Upon removal of these proteins, the Ca^{2+} and/or Cl^- requirements are significantly increased for optimal oxygen evolution activity *in vitro* (4). Because the cyanobacterium *Synechocystis* sp. PCC 6803 is especially amenable to genetic manipulation, directed mutant studies have provided a large amount of functional information on the cyanobacterial PS II extrinsic proteins. Burnap and Sherman (13) reported that the *psbO* deletion mutant (Δ*psbO*) is photoautotrophic, and therefore cyanobacterial PsbO is not essential for oxygen evolution activity, unlike its plant counterpart (13). In a study of *psbU* and *psbV* deletion mutants (Δ*psbU* and Δ*psbV*, respectively), Shen *et al.* (14) suggested that PsbU maintained the maximum affinity of PS II for

Ca^{2+} and Cl^-. Deletion of *psbV* resulted in significant defects in PS II function; $\Delta psbV$ cells exhibited a slower photoautotrophic growth rate and 40% oxygen evolution activity compared with wild type (15). From the biochemical and genetic studies, Shen and co-workers (4, 16, 17) proposed that PsbU and PsbV in cyanobacteria were similar in function to PsbQ and PsbP in chloroplasts. Accordingly, an evolutionary model was described in which PsbQ and PsbP replaced PsbU and PsbV at some point during evolution from the ancestral cyanobacterium to chloroplasts (2, 17, 18). However, this model is somewhat unsatisfactory, because no similarity in the predicted primary sequences or the crystallographic structures was found between these sets of proteins (PsbU/PsbV *versus* PsbP/PsbQ) (2, 19–24).

Subsequent to these studies, proteomic analysis of highly purified PS II complexes isolated from a HT3 strain of *Synechocystis* 6803 identified a novel protein homologous to chloroplast PsbQ (5). Further analysis of the *Synechocystis* 6803 genome identified a protein homologous to PsbP (6). Initial functional analyses showed that both PsbP and PsbQ have regulatory role(s) in the oxygen evolution machinery, since the deletion of the corresponding genes results in retarded growth under Ca^{2+}- or Cl^--limited conditions (6). The PsbQ protein was found to be associated with PS II complexes stoichiometrically, whereas the PsbP protein is substoichiometric in PS II (6). Therefore, PS II complexes from the cyanobacterium *Synechocystis* 6803 contain five associated extrinsic proteins: PsbO, PsbP, PsbQ, PsbU, and PsbV.

A 20-kDa protein homologous to plant PsbQ was found in PS II complexes purified from a primitive red alga, *Cyanidium caldarium*, which also contains the PsbU and PsbV proteins (18). Careful genome analysis of cyanobacteria showed that most cyanobacterial genomes contain genes homologous to *psbP* and *psbQ* (25), consistent with our findings for *Synechocystis* 6803 (5, 6). These new findings challenge the proposed model that PsbP and PsbQ in plants have functionally replaced PsbU and PsbV from cyanobacteria. Indeed, recent studies of the PS II extrinsic proteins have focused on more rigorously assigning functions to each of the five extrinsic proteins in cyanobacterial PS II (26, 27). The function of PsbU was reexamined, and this new analysis of $\Delta psbU$ indicates that PsbU in cyanobacterial PS II functions to support a stable structural architecture of the water-splitting system (26). Summerfield *et al.* (27) extended our findings on PsbQ, showing stringent requirements for PsbQ *in vivo*. They reported that the $\Delta psbQ$ mutant had impaired growth at elevated temperatures. They also combined the $\Delta psbQ$ mutation with other PS II extrinsic protein mutations in *Synechocystis* 6803 (27). The double deletion mutant $\Delta psbQ\Delta psbV$ was not able to grow photoautotrophically, whereas the $\Delta psbQ\Delta psbO$ and $\Delta psbQ\Delta psbU$ mutants showed exacerbated growth defects relative to the single mutants, especially in medium lacking Ca^{2+} and Cl^-. Accordingly, they concluded that PsbQ has a role in optimizing PS II activity in *Synechocystis* 6803 cells, which is absolutely required under specific physiological conditions.

Although these recent studies highlight a role of PsbQ in cyanobacterial PS II, the reported structural models of cyanobacterial PS II complexes from *Thermosynechococcus elongatus* and *Thermosynechococcus vulcanus* do not contain PsbQ (19, 23, 24). Thus, it is not known how PsbQ interacts with the other extrinsic proteins (PsbO, PsbU, and PsbV) nor how this may contribute to the water oxidation reaction. In this report, we specifically focus on the localization and the function of PsbQ within the water oxidation machinery of cyanobacterial PS II. Our results demonstrate that PsbQ associates with the luminal surface of PS II despite differences in its biochemical properties relative to the other extrinsic proteins, PsbO, PsbU, and PsbV. Functional analyses of the water oxidation machinery in the *psbQ* deletion mutant ($\Delta psbQ$) show significant effects on the donor side of PS II, including destabilization of PsbV in the complex and a more exposed catalytic center. From these data, we propose that the major function of PsbQ is to stabilize the PsbV protein on the luminal surface of PS II and protect the catalytic $Mn_4\text{-}Ca_1\text{-}Cl_x$ cluster from exogenous reductants.

EXPERIMENTAL PROCEDURES

Cyanobacterial Culture Conditions—Wild type, $\Delta psbQ$, and $\Delta psbV$ (6) strains of *Synechocystis* 6803 were grown in normal BG 11 medium (28). The HT3 strain of *Synechocystis* 6803 and $\Delta psbQ$HT3 mutant (6) were grown in BG 11 medium supplemented with 2 mM glucose and 50 μg/ml kanamycin (for HT3) or spectinomycin (for $\Delta psbQ$) at 30 °C under 50 μmol of photons $m^{-2} s^{-1}$. HT3 strain was kindly provided by Dr. T. Bricker (29).

Isolation of Thylakoid Membranes and Purification of PS II Complexes—Thylakoid membrane and PS II complexes were isolated as described in Ref. 5 and resuspended in MMCG solution, containing 50 mM MES-NaOH (pH 6.0), 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 25% glycerol.

Isolation of Right Side-out and Inside-out Vesicles—To isolate thylakoid membranes containing predominantly right side-out vesicles (30), cells in MMCG solution were broken as described in Ref. 5 and collected at $36,000 \times g$ after the removal of unbroken cells. For the isolation of thylakoid membranes containing mostly inside-out vesicles (30), cells suspended in 50 mM MES-NaOH (pH 6.0), 10 mM $MgCl_2$, 5 mM $CaCl_2$ were passed through a French pressure cell twice at 100 megapascals. After removal of unbroken cells by centrifugation at $3,000 \times g$, the resulting vesicles were collected at $160,000 \times g$. Both types of vesicles were suspended in MMCG solution in the final step of preparation. No protease inhibitors were added during these isolations. The lysozyme treatment was omitted to avoid possible proteolytic degradation. Instead, the samples were thoroughly kept cool and processed quickly.

Fractionation of Thylakoid Membrane—Triton X-114 phase partitioning of thylakoid membrane was performed essentially as described in Ref. 31 at 100 μg of Chl/ml. Triton X-114 was purchased from Sigma.

Protease Treatment—Stock solution of trypsin (6,750 units/ml; Sigma; pancreatic type II crude) was added to 1 ml of thylakoid membrane suspension (100 μg of Chl/ml) to 0, 34, 68, and 135 units/100 μg of Chl (equivalent to around 4 mg of protein). The mixtures were incubated at 30 °C for 10 min at pH 6.0. Note that because the pH was not optimal for trypsin activity, the digestion was performed at 30 °C. The reactions were

stopped by adding one-third volume of denaturing solution (20 mM EDTA, 5% lithium dodecyl sulfate, 40 mM dithiothreitol, 172 mM Tris, pH 6.8, and 0.5 M sucrose) and cooling to 0 °C.

SDS-PAGE and Protein Detection—Electrophoresis and immunodetection were performed as described in Ref. 32, using a 18–24% gradient acrylamide, 6 M urea SDS-PAGE system. For immunodetection, PsbQ was detected using specific antisera against cyanobacterial PsbQ (6). Bands were visualized using enhanced chemiluminescence reagents (WestPico; Pierce) on a Fujifilm LAS-1000 Plus imager (Fujifilm, Stamford, CT). The c-type heme in cytochrome c_{550} was also detected using WestFemto (Pierce) on a LAS-1000 Plus imager after the transfer of proteins onto polyvinylidene fluoride (PVDF) membrane (Immobilon P; Millipore, Billerica, MA) (33).

Oxygen Evolution Assay—Steady state oxygen evolution was measured on a Clark-type electrode in the presence of 1 mM potassium ferricyanide and 0.5 mM 2,6-dichloro-*p*-benzoquinone as electron acceptors at 2 μg of Chl/ml in MMCS buffer containing 50 mM MES-NaOH, (pH 6.0), 10 mM $MgCl_2$, 5 or 20 mM $CaCl_2$ and 0.5 M sucrose. To assess the effect of NH_2OH, oxygen evolution was measured after PS II complexes (2.0 μg of Chl/ml in MMCG buffer, 20 mM $CaCl_2$) were incubated for 1 h on ice in the dark in the presence of variable concentrations of NH_2OH (34). Flash oxygen yield measurements were measured using a bare platinum electrode as described in Ref. 26.

Fluorescence Kinetics—Q_A reoxidation kinetics was measured at room temperature using a double-modulation fluorometer, FL-3320 (Photon System Instruments, Brno, Czech Republic) with FluorWin software (version 3.6.3.3). The cell concentration for each sample was adjusted to $OD_{730} = 0.08$ (~2 μg of Chl/ml) measured on a DW2000 spectrophotometer (SLM-Aminco, Urbana, IL). The samples were dark-adapted for 3 min prior to measuring.

Optical Measurements—Chl *a* concentration was determined by the method of Porra *et al.* (35).

RESULTS

Cyanobacterial PsbQ Is a Luminal Protein with a Putative Lipid Anchor—We have previously postulated that PsbQ in cyanobacteria is a luminally targeted lipoprotein (6). Indeed, cyanobacterial PsbQ was not removed from PS II by biochemical treatments (1 M $CaCl_2$ or 1 M Tris-HCl, pH 8.0) that removed the other extrinsic proteins (PsbO, PsbU, and PsbV) (5). This result was quite different from the characteristics of chloroplast PsbQ, which is removed by high salt treatment (36).

Triton X-114 partitioning, a well established method to separate hydrophobic and hydrophilic proteins (37), was used to assess the hydrophobicity of the mature cyanobacterial PsbQ protein. Thylakoid membranes were subjected to Triton X-114 partitioning, and the proteins were fractionated into a hydrophobic fraction (Triton X-114 fraction; Fig. 1, *lane 3*), an aqueous fraction (*lane 4*), and an insoluble fraction (very hydrophilic; *lane 5*). Upon Coomassie staining, the separate fractions showed different polypeptide profiles. As expected, the hydrophilic phycobiliproteins partitioned into the aqueous and insoluble fractions (Fig. 1, Coomassie staining and no staining, *lanes 4* and *5*). Heme staining was used to detect PsbV (cytochrome c_{550}, apparent molecular mass of 22 kDa), which fractionated
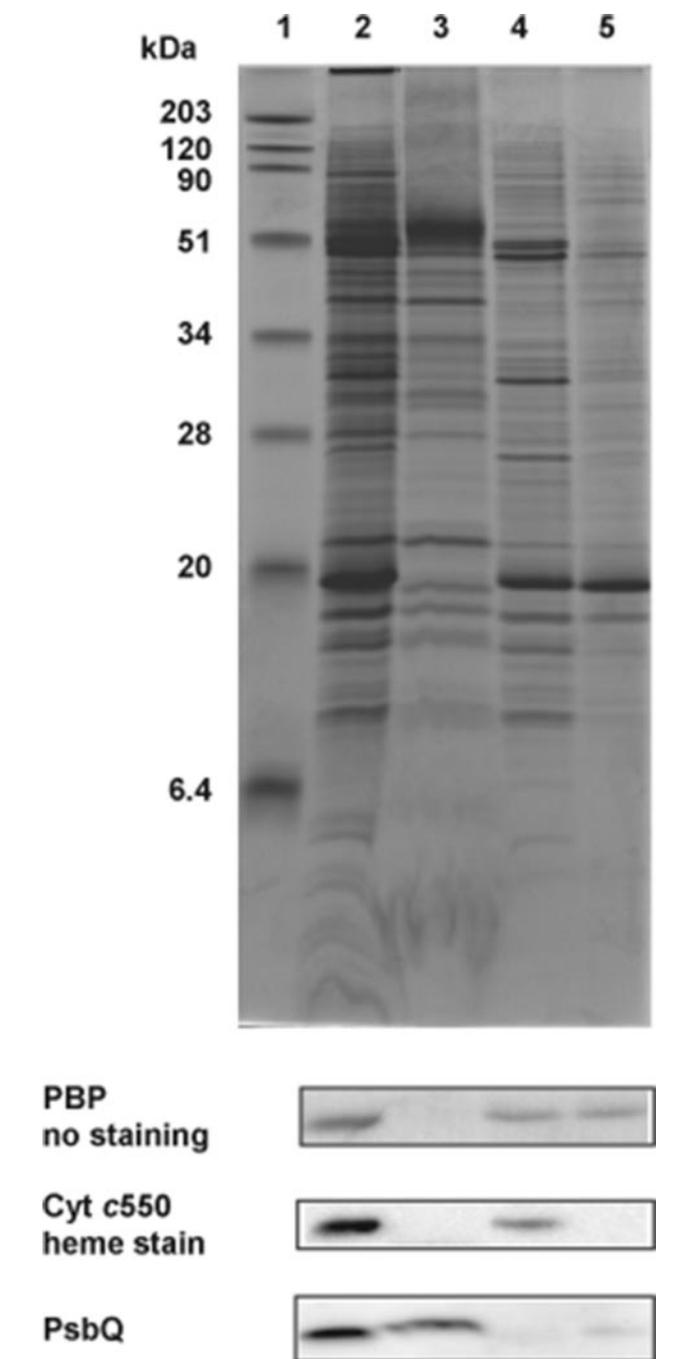

FIGURE 1. **Triton X-114 mediated phase partitioning of thylakoid membranes.** *Lane 1*, molecular weight standards; *lane 2*, thylakoid membrane; *lane 3*, Triton X-114 phase; *lane 4*, aqueous phase; *lane 5*, insoluble fraction. *Top*, Coomassie-stained gel; *second panel*, after transfer on PVDF membrane, no staining; *third panel*, heme staining; *bottom panel*, immunodetection using antiserum against PsbQ. Each fraction was adjusted to the same sample volume (40 μl), which was equivalent to 4 μg of Chl of the initial thylakoid membrane preparation.

into the aqueous phase (Fig. 1, heme staining, *lane 4*). This result is in agreement with previous reports that PsbV is removed by high salt treatment (4, 5). Unlike these hydrophilic proteins, PsbQ partitioned into the hydrophobic fraction (Fig. 1, PsbQ, *lane 3*).

Although PsbQ partitioned into the hydrophobic phase (Fig. 1), this protein has no predicted hydrophobic domain and is expected to be exposed on one side of the thylakoid membrane.
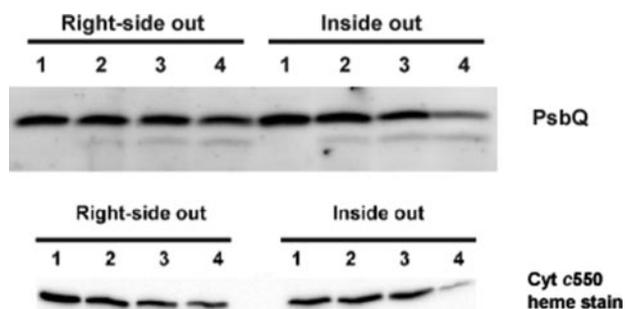
FIGURE 2. **Trypsin treatment of inside-out and right side-out membrane vesicles.** After treatment with trypsin for 10 min at 30 °C, the membranes were fractionated by electrophoresis and visualized by immunodetection using antiserum against PsbQ after blotting onto a PVDF membrane (*top*) and heme staining (*bottom*). *Lane 1*, no trypsin; *lane 2*, 34 units of trypsin to 4 mg of protein (100 μg of Chl); *lane 3*, 68 units of trypsin to 4 mg of protein (100 μg of Chl); *lane 4*, 135 units of trypsin to 4 mg of protein (100 μg of Chl). Samples equivalent to 3 μg of Chl (120 μg of protein) were loaded in each *lane*.

Right side-out and inside-out thylakoid membranes were prepared and subjected to limited digestion by trypsin to assess the topology of PsbQ (Fig. 2). PsbQ was not digested in the right side-out membrane samples but was digested in the inside-out membrane samples, similar to the digestion pattern for the luminal protein PsbV (cytochrome c_{550}). This result demonstrates that PsbQ is located on the luminal side of thylakoid membrane. Altogether, the data from the Triton X-114 partitioning and the trypsin digestion experiments are consistent with the previous prediction that PsbQ is targeted to the thylakoid lumen and cleaved by signal peptidase II to yield an N-terminal cysteine, which is modified with a lipid anchor (6).

The PsbV Protein Partially Dissociates from ΔpsbQHT3 PS II Complexes—The polypeptide profiles of HT3 and ΔpsbQHT3 PS II complexes are shown in Fig. 3. As expected, the PsbQ protein is absent from the ΔpsbQHT3 PS II complexes. Also, PsbV was partially lost in ΔpsbQHT3 PS II, indicating that, in the absence of PsbQ, the structure of the water oxidation machinery becomes labile. The fact that PsbV, a luminal PS II protein, is affected by the absence of PsbQ is also consistent with the luminal localization of PsbQ determined during the preceding experiments.

The ΔpsbQ Mutant Has Defects in PS II Water Oxidation—To address the contribution of PsbQ to the water oxidation process, fluorescence kinetics measurements were performed using cells grown in normal BG11. Because PsbV became labile in PS II upon the absence of PsbQ, the ΔpsbV mutant was also assayed for comparison. For samples at the same chlorophyll concentration, the F_o value for ΔpsbQ cells was essentially the same as that of wild type cells (0.735 ± 0.0802 *versus* 0.715 ± 0.0805; S.D., n = 5–6). This is consistent with previous data showing no significant difference in the relative amounts of PS II between wild type and ΔpsbQ cells (6). Whereas the F_o value of ΔpsbV cells is significantly increased relative to wild type (1.08 ± 0.136; S.D., n = 5–6), indicating an increase in impaired PS II complexes. The normalized variable fluorescence yield in ΔpsbQ was also comparable with that in wild type (0.742 ± 0.0864 *versus* 0.694 ± 0.0379; S.D., n = 5–6), whereas that of ΔpsbV was much lower (0.307 ± 0.121; S.D., n = 5–6). The fluorescence decay kinetics for cells after a single saturating flash, which represents the reoxidation kinetics of Q_A^- in PS II,
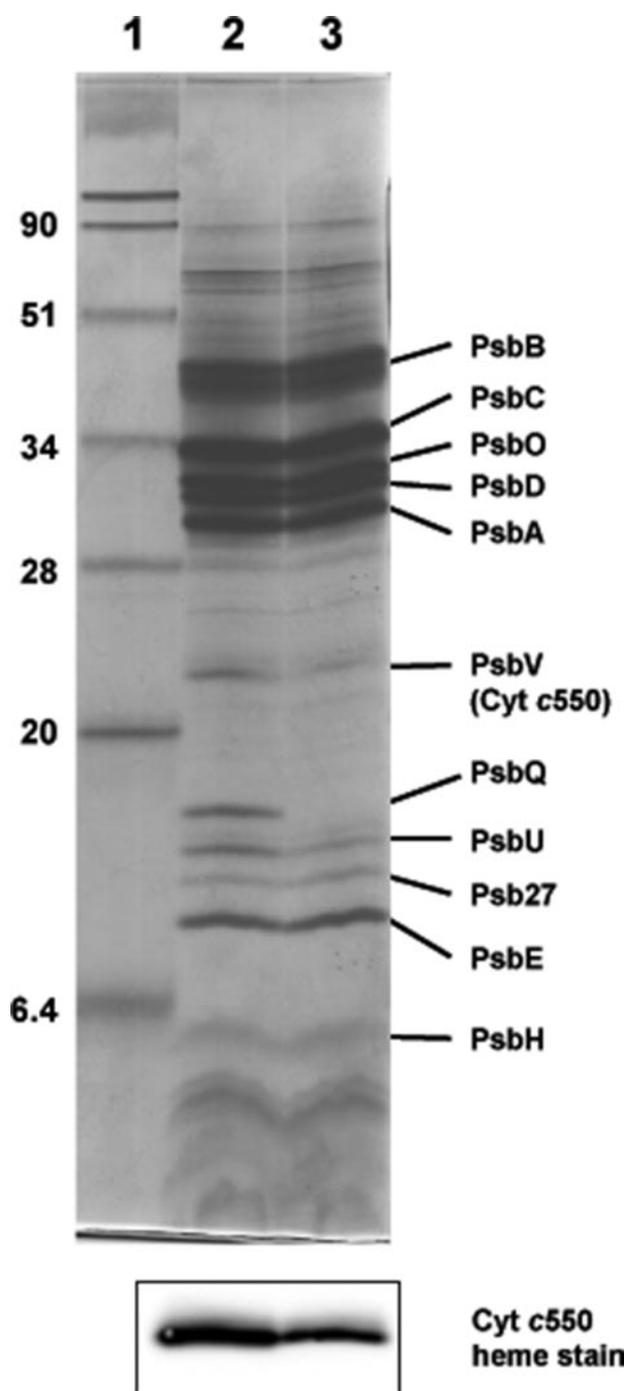

FIGURE 3. **Polypeptide profiles of purified PS II.** *Top*, *lane 1*, molecular weight standards; *lane 2*, PS II complexes purified from HT3; *lane 3*, PS II complexes purified from ΔpsbQHT3. *Bottom*, heme staining after transfer on PVDF membrane. Each *lane* contains 5 μg of Chl.

are shown in Fig. 4. Qualitatively, the ΔpsbQ mutant has a decay curve intermediate to that of wild type and the ΔpsbV mutant. To quantitatively compare the fluorescence decay curves for all strains, they were fit with three components of different decay half-times (Table 1). The first phase reports on the electron transfer from Q_A to Q_B (38–40). The second phase represents the turnover of plastoquinone molecules at the Q_B site (41). The last component with the longest half-time is the oxidation of Q_A by PS I (42). It is noteworthy that the decay half-time of the
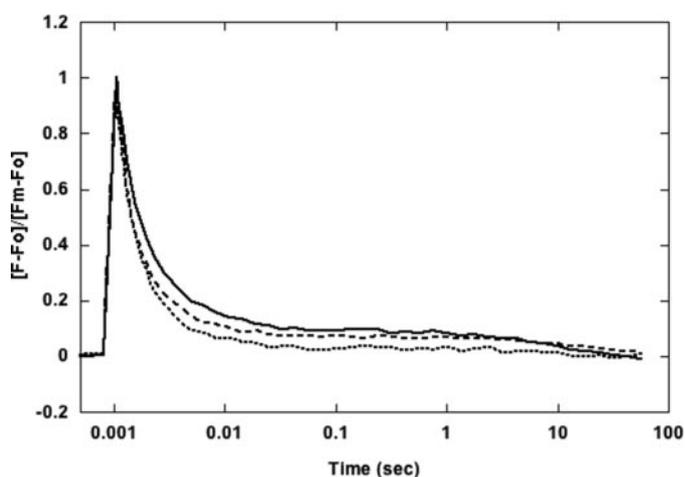
FIGURE 4. **Kinetics of Q_A^- reoxidation.** Fluorescence was measured on samples at $OD_{730} = 0.08$ ($\sim$2 μg of Chl/ml) at room temperature after 3 min of dark incubation. *Solid line*, wild type; *dashed line*, $\Delta psbQ$; *dotted line*, $\Delta psbV$.

TABLE 1

Exponential decay components in Q_A^- reoxidation kinetics

Fluorescence decay kinetics was measured in wild type, $\Delta psbQ$, and $\Delta psbV$ as in Fig. 4 and was deconvoluted to three exponential decay components. Curve fitting was performed using KaleidaGraph software (version 3.6 for Macintosh; Synergy Software, Reading, PA), with a Levenberg-Marquardt regression algorithm. The curves were better fit with three exponential decay components rather than two exponential decay components. Numbers in parentheses are S.E. ($n = 6$ or 7).

	Decay half-time	Relative amount
		%
Wild type	307 $\pm$ 21.1 μs	94 $\pm$ 0.87
	3.28 $\pm$ 0.346 ms	4.7 $\pm$ 0.59
	9.63 $\pm$ 1.18 s	1.3 $\pm$ 0.30
$\Delta psbQ$	244 $\pm$ 14.3 μs	97 $\pm$ 0.49
	3.29 $\pm$ 0.704 ms	2.3 $\pm$ 0.36
	13.5 $\pm$ 1.87 s	0.64 $\pm$ 0.15
$\Delta psbV$	250 $\pm$ 17.5 μs	97 $\pm$ 0.57
	3.17 $\pm$ 0.544 ms	2.7 $\pm$ 0.46
	14.9 $\pm$ 5.41 s	0.25 $\pm$ 0.13

fastest component in $\Delta psbQ$ was 20% faster than that in wild type; furthermore, the relative amounts of each component in $\Delta psbQ$ differed from those in wild type. This result is consistent with that observed in the $\Delta psbV$ mutant in which the water oxidation process is highly modified.

Flash oxygen yield measurements were also conducted to assay the function of the water oxidation machinery in the absence of PsbQ. $\Delta psbQ$ and $\Delta psbV$ cells showed a characteristic but somewhat modified period four oscillation. The relative oxygen yields after first and second flashes were slightly larger in $\Delta psbQ$ cells than in wild type cells but smaller than in $\Delta psbV$ (data not shown).

The distribution of S-states after 10 min of dark adaptation was somewhat perturbed in $\Delta psbQ$ cells relative to wild type cells. $\Delta psbQ$ cells exhibited a higher fraction of the S_3-state (4.5 ± 0.18 *versus* $2.3 \pm 0.18\%$; S.E., $n = 4$–8) and a lower fraction of the S_0-state (32 ± 0.83 *versus* $37 \pm 0.81\%$; S.E., $n = 4$–8) The distribution in $\Delta psbV$ cells was more perturbed relative to that in wild type cells with an increased population of S_2- and S_3-states and a lower fraction of S_0- and S_1-states (data not shown). The yield itself was also affected, in that the averaged amplitude after damping (flash number 13–16) in $\Delta psbQ$ cells was typically 90% of that in wild type cells ($n = 5$) but still larger than that in $\Delta psbV$ cells (24% of that in wild type; $n = 6$).
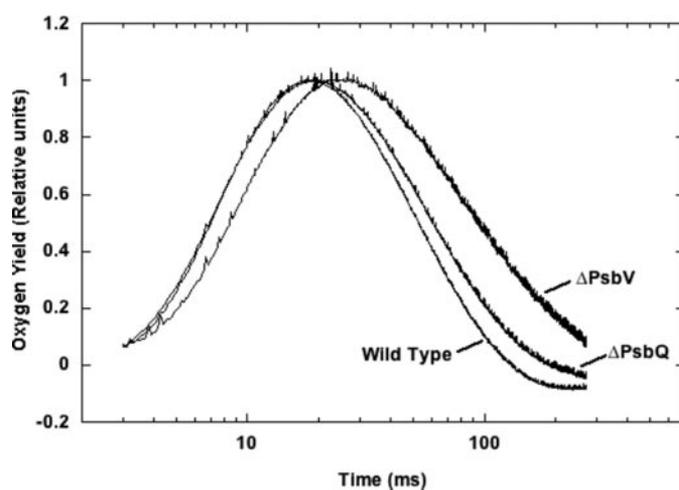

FIGURE 5. **Kinetics of oxygen release from cells measured on a bare platinum electrode.** Cells on the electrode surface were dark-adapted for 10 min and then subjected to illumination with a train of flashes at 3.3 Hz. The oxygen signal produced by the third flash illumination is shown. The signals were normalized to the highest amplitude in each strain.

TABLE 2

Oxygen evolution activity in the presence of 5 or 20 mM $CaCl_2$

The rate of oxygen evolution was assayed in the presence of 1 mM ferricyanide and 0.5 mM DCBQ in MMCS solution. The final concentration of $CaCl_2$ is shown. Rates are given in μmol of O_2 mg of $Chl^{-1} h^{-1}$.

	HT3 PS II	$\Delta psbQ$HT3 PSII
	$\mu mol\ O_2\ mg\ of\ Chl^{-1}\ h^{-1}$	
5 mM $CaCl_2$	1940 $\pm$ 153 ($n = 3$)	800 $\pm$ 0.0 ($n = 3$)
20 mM $CaCl_2$	1950 $\pm$ 212 ($n = 2$)	1160 $\pm$ 40.3 ($n = 3$)

Fig. 5 shows the kinetics of oxygen release from cells poised in the S_3-state, as detected using a bare platinum electrode for wild type, $\Delta psbQ$, and $\Delta psbV$ cells (14, 43). The rise kinetics for wild type and $\Delta psbQ$ cells were identical, giving the same peak time ($\sim$17 ms following the third flash), whereas $\Delta psbV$ cells showed a slower rise time and $\sim$7-ms slower peak time than the other strains (Fig. 5). Interestingly, the decay half-time of the oxygen release curve was different for wild type and $\Delta psbQ$ cells (29.5 and 39 ms, respectively). In the $\Delta psbQ$ cells, the decay was decelerated, but not to the extent of that in $\Delta psbV$ cells (62.8 ms). Here again, the $\Delta psbQ$ mutant displays a phenotype intermediate to that of wild type and $\Delta psbV$. Although this signal has been used previously as an indirect measurement of S_3- to S_0-state advancement (14, 43), other factors, including long oxygen diffusion pathways to the electrode and oxygen consumption by the cells, also contribute to its lifetime.

The Structural Integrity of the PS II Water Oxidation Complex Is Compromised in the Absence of PsbQ—It has been reported that the removal of PsbP and PsbQ from higher plant PS II membrane preparations causes a remarkable decrease in the oxygen evolution rate and an increase in the Ca^{2+} and Cl^- requirement for activity and exposes the oxidizing side of PS II to exogenous reductants, such as NH_2OH (12, 44). Table 2 shows the steady state oxygen evolution activities of isolated PS II complexes from HT3 and $\Delta psbQ$HT3. The activity of $\Delta psbQ$HT3 PS II complexes was lower than that of HT3 even in the presence of 20 mM $CaCl_2$ (60% of HT3 PS II). Whereas HT3 PS II evolved oxygen at the same rate in the presence of 5 and 20 mM $CaCl_2$, the rate of oxygen evolution by $\Delta psbQ$HT3 PS II
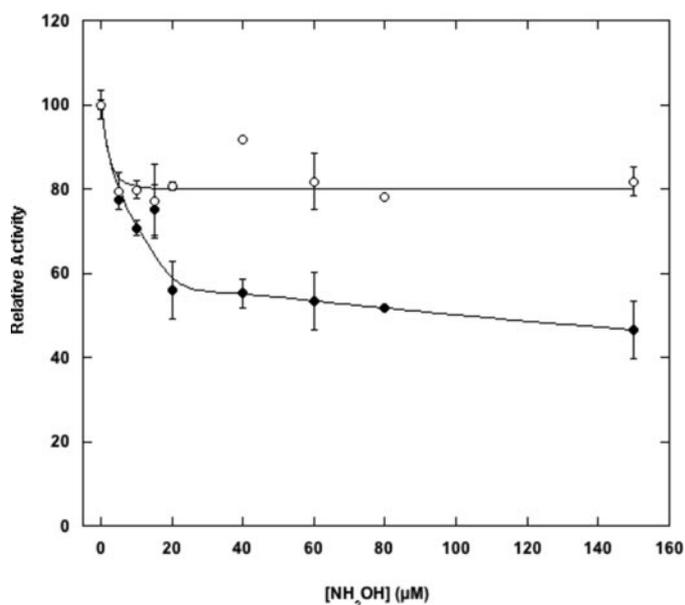
FIGURE 6. **Accessibility of NH₂OH to the donor side of PS II.** The PS II complexes (2 μg Chl/ml) were incubated with various concentrations of NH₂OH for 1 h on ice in the dark. The rate of oxygen evolution supported by saturated light was measured in the presence of 1 mM ferricyanide and 0.5 mM 2,6-dichloro-*p*-benzoquinone. The oxygen evolution activity at 0 μM NH₂OH were 1130 and 464 μmol of O₂ mg of Chl^{-1} h^{-1} for HT3 and $\Delta psbQ$HT3 PS II, respectively.

with 5 mM CaCl₂ decreased to 70% of its activity in the presence of 20 mM CaCl₂. Thus, PS II complexes lacking PsbQ are more sensitive to the amount of CaCl₂ in the assay buffer relative to the control.

To further analyze the structural integrity of the isolated PS II complexes, the effect of hydroxylamine on steady state oxygen evolution activity was measured (Fig. 6). In the presence of NH₂OH, the rate of oxygen evolution in HT3 PS II decreased by around 20% independent of the NH₂OH concentration tested. In contrast, the oxygen evolution activity of $\Delta psbQ$HT3 PS II decreased in a clearly NH₂OH concentration-dependent manner, with a steep decrease up to 20 μM NH₂OH and a gradual decrease above 20 μM NH₂OH. These results show that the water oxidation machinery in HT3 PS II is largely protected from the small reductant NH₂OH, whereas that of $\Delta psbQ$HT3 PS II is highly exposed and therefore easily accessed and damaged by NH₂OH.

DISCUSSION

The current models for PS II extrinsic proteins include five proteins in cyanobacteria (PsbO, PsbU, PsbV, PsbP, and PsbQ) and three proteins in higher plants (PsbO, PsbP, and PsbQ) (6). The PsbO protein, which is shared by both systems, may have a common function in cyanobacteria and plants. However, the PsbU and PsbV proteins have a specialized function in cyanobacteria, whereas PsbP and PsbQ have a modified function between the two systems. Therefore, it is necessary to reevaluate the roles of each of these proteins in the water oxidation reaction. The current study has focused on the localization and function of cyanobacterial PsbQ.

Determination of the localization of PsbQ within the cyanobacterial PS II complex is critical for understanding the roles

of all of the associated extrinsic proteins. Structural studies have not yet resolved the PsbQ protein in PS II (19, 23, 45, 46). Cyanobacterial PsbQ associates with PS II complexes but shows different biochemical properties than higher plant PsbQ, since it is not removed by 1 M CaCl₂ or 1 M Tris (5, 6). In this work, the physical properties of cyanobacterial PsbQ were investigated using Triton X-114 partitioning. Our results show that PsbQ fractionated into the hydrophobic Triton X-114 phase (Fig. 1). This result is consistent with our previous prediction from sequence analysis that PsbQ is a lipoprotein (6). This kind of modification has been reported previously in *Synechocystis* 6803 for the periplasmic protein NrtA (47). Thus, it is reasonable to assume that the N-terminal lipid modification causes PsbQ to partition into the hydrophobic Triton X-114 phase and anchors PsbQ to the membrane such that it is not removed by 1 M CaCl₂ or 1 M Tris (5).

The fact that cyanobacterial PsbQ is a lipoprotein may explain why PsbQ is present in HT3 PS II but not in the current crystallographic model (19, 23, 24). HT3 PS II was purified under mild detergent conditions (33), and it is expected that the lipid bound to the N terminus of PsbQ should remain anchored to the hydrophobic domain of PS II. However, the prevailing procedure to purify membrane protein complexes suitable for crystallization includes a step(s) to remove excess lipids (33, 48–50). Thus, when the lipids around the PS II complex were removed during purification, it is conceivable that PsbQ was also removed or could no longer anchor to the lipid-depleted PS II complex. Tll2057, the PsbQ homologue in *T. elongatus* used for PS II crystallographic studies, (available on the World Wide Web at www.kazusa.or.jp/cyano/Thermo/index.html), is also predicted to contain a lipoprotein signal peptide. The functional significance of the N-terminal lipid anchor in cyanobacterial PsbQ is unclear, since PsbQ in plants lacks this modification.

To confirm the luminal localization of cyanobacterial PsbQ, thylakoid membrane vesicles of opposite orientations were subjected to trypsin digestion. PsbQ was digested by trypsin in the inside-out membrane samples, whereas it was protected from digestion in the right side-out membrane samples, as was the *bona fide* luminal protein PsbV (cytochrome c_{550}). We have reported previously that upon trypsin digestion of right side-out membranes prepared in this way, the luminal PsbO protein is protected from digestion, whereas the cytoplasmically exposed PsaD protein is degraded. The opposite digestion profiles were observed for the inside-out prepared membranes (30). Our current results clearly indicate that PsbQ localizes to the luminal side of thylakoid membrane and excludes the possibility of the presence of a transmembrane domain in cyanobacterial PsbQ. Altogether, these data confirm that PsbQ closely associates to the luminal side of cyanobacterial PS II complexes (Fig. 2) (5), and it can be concluded that PsbQ is a component of the water oxidation complex in cyanobacterial PS II.

Based on the above mentioned localization of PsbQ, the role of PsbQ in relation to the water oxidation reaction was assessed. The variable yield of the fluorescence (($F_m - F_o)/F_o$) in $\Delta psbQ$ was comparable with that of wild type, whereas the yield in $\Delta psbV$ was much smaller than that of wild type (typically less

than 45%). The rate of Q_A reoxidation was accelerated in $\Delta psbQ$ as well as $\Delta psbV$ with the decay half-time of the major, fastest component 20% faster in the mutant cells compared with that of wild type cells (Fig. 4 and Table 1). Although Q_A is localized on the reducing side of PS II, defects on the oxidizing side of PS II directly cause an acceleration of Q_A reoxidation (51). This result indicates that the absence of PsbQ affects the stability of the water oxidation complex.

Flash yield oxygen measurements showed a slightly altered S-state distribution for $\Delta psbQ$ relative to wild type cells, consistent with the lower oxygen evolution activity observed in $\Delta psbQ$ cells (6, 27). The rise kinetics of oxygen release from cells poised in the S_3-state in $\Delta psbQ$ cells was identical to that of wild type cells (Fig. 5). Although the oxygen signal after the third flash is not a direct measurement of oxygen release from the enzyme, this signal has been correlated to the kinetics of oxygen release from the S_3-state, assuming that the diffusion rates of oxygen from different cells to the electrode are similar (14, 43). Whereas $\Delta psbQ$ cells behaved similarly to wild type cells for the rise kinetics of oxygen release, the decay kinetics of oxygen release in $\Delta psbQ$ cells were slower compared with wild type but not to the extent of that in $\Delta psbV$ cells (Fig. 5). The biological significance of the decay part of the oxygen release curve is not known, and additional processes (*i.e.* oxygen diffusion to the electrode and oxygen consumption by the cells) contribute to this part of the signal.

The absence of PsbQ had specific consequences on the extrinsic protein PsbV (cytochrome c_{550}). Whereas the Chl a-normalized amount of PsbV in $\Delta psbQ$ cells was comparable with that in wild type cells (measured by heme staining; data not shown), there was a significant decrease in the amount of PsbV associated with PS II complexes purified from the $\Delta psbQ$HT3 mutant (Fig. 3). The absence of PsbQ did not result in the complete removal of PsbV, and this is consistent with previous reports that the $\Delta psbQ$ mutant grew faster than $\Delta psbV$ mutant in both normal medium and Ca^{2+}- or Cl^--depleted medium (6). From these results, we conclude that PsbQ is in close proximity to the PsbV protein within PS II and functions to stabilize the extrinsic proteins in the PS II water oxidation complex. These results suggest that a common role for PsbQ in cyanobacteria and plants is to stabilize other PS II extrinsic proteins and modulate the Cl^- requirement for oxygen evolution activity. However, the specific effect on PsbV stability in cyanobacterial PS II upon the loss of PsbQ is somewhat surprising, because higher plant PS II complexes do not contain PsbV.

The absence of PsbQ leads to low water oxidation activity in isolated PS II and the higher requirement of Ca^{2+} and Cl^- for oxygen evolution (Table 2). This is consistent with the observed effect on PsbV in the $\Delta psbQ$ mutant. To further probe the stability of the water oxidation machinery in PS II complexes lacking PsbQ, the effect of NH_2OH was measured. Ghanotakis *et al.* (12) have shown that if PsbP and PsbQ are removed from intact PS II membranes from higher plants, the small reductant NH_2OH causes extensive damage to the water-oxidizing side of PS II. Thus, PsbP and PsbQ, along with PsbO, shield the water oxidization machinery of plant PS II from the luminal space, and once these proteins are removed, the accessibility of NH_2OH to the Mn_4-Ca_1-Cl_x cluster increases significantly. The

same effect was observed in isolated PS II complexes from the $\Delta psbQ$HT3 mutant. $\Delta psbQ$HT3 PS II was considerably more sensitive to NH_2OH, whereas only a limited effect was observed for HT3 PS II (Fig. 6). These results highlight the role of PsbQ in stabilizing the components of the water oxidation machinery in cyanobacterial PS II.

In conclusion, PsbQ associates with the luminal side of cyanobacterial PS II complexes and participates in the water oxidation reaction. PsbQ is important for stabilizing PsbV within the PS II complex, and the majority of the defects described for $\Delta psbQ$ can be explained by a partial loss of PsbV. However, PsbQ must have a role beyond that of stabilizing PsbV, because the double deletion mutant $\Delta psbQ\Delta psbV$ cannot grow photoautotrophically, whereas the respective single mutants can grow photoautotrophically (27). It is possible that PsbQ contributes to the stabilization of the other extrinsic proteins on the luminal side of PS II, and the absence of both PsbQ and PsbV results in such an instability of the water oxidation machinery that it can no longer support photoautotrophic growth. Thus, PsbQ is an important extrinsic protein in cyanobacterial PS II, which contributes to the protection of the catalytic Mn_4-Ca_1-Cl_x cluster of the water oxidation machinery.

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