An Independent Role of Cytochrome *c*-550 in Cyanobacterial Photosystem II As Revealed by Double-Deletion Mutagenesis of the *psb*O and *psb*V Genes in *Synechocystis* sp. PCC 6803[†]

Jian-Ren Shen,*[,‡] Robert L. Burnap,[§] and Yorinao Inoue[‡]

Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan, and Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, Oklahoma 74078

Received May 15, 1995; Revised Manuscript Received July 10, 1995[⊗]

ABSTRACT: Cytochrome (cyt) *c*-550 and the 33 kDa protein are two extrinsic components that function in maintaining oxygen evolution in cyanobacterial cells. Deletion of either of the two components has been shown to result in cyanobacterial phenotypes that are still capable of photoautotrophic growth albeit with a reduced rate. In order to study the function of cyt *c*-550 in cyanobacterial photosystem II (PSII) and its possible interaction with the 33 kDa extrinsic protein, we constructed a mutant lacking both cyt *c*-550 and the 33 kDa protein by inactivating the *psb*V and *psb*O genes simultaneously in a cyanobacterium, *Synechocystis* sp. PCC 6803. The resultant double-deletion mutant was unable to grow photoautotrophically and showed almost no oxygen-evolving activity (less than 10% of the wild type). This residual activity was also lost rapidly upon illumination, suggesting an increased sensitivity of the mutant cells toward photoinhibition. Thermoluminescence measurements indicated that the mutant virtually cannot undergo normal charge accumulation (S-state transitions) leading to oxygen evolution. Herbicide-binding and Western blot analyses showed that the mutant accumulates the PSII complex to an extent of only 20% of that in wild-type cells. Combined with previous results, the present results indicated that cyt *c*-550 supported oxygen evolution in the single-deletion mutant lacking the 33 kDa protein alone and *vice versa*. Thus, both cyt *c*-550 and the 33 kDa protein function independently in maintaining cyanobacterial oxygen-evolving activity *in vivo*, and both of them are required for the optimal activity. This not only provides another conclusive line of evidence for the concept that cyt *c*-550 functions in cyanobacterial PSII oxygen evolution but also reveals a functional difference between this extrinsic cyt in cyanobacterial PSII and the extrinsic 23 kDa protein in higher plant PSII, as the 23 kDa protein alone cannot support oxygen evolution in the absence of the 33 kDa protein.

Photosynthetic oxygen evolution takes place in a distinct integral membrane−protein complex referred to as photosystem II (PSII).[1] The PSII complex contains at least seven membrane-spanning subunits that are commonly found in cyanobacteria and higher plants: the reaction center proteins D1 and D2, the intrinsic chlorophyll-binding proteins CP47 and CP43, the α and β subunits of cyt *b*-559 (9 and 4.5 kDa, respectively), and the *psb*I gene product (5 kDa) [for reviews, see Erickson and Rochaix (1992) and Ikeuchi (1992)]. In addition, three water-soluble proteins are attached to the surface of the membrane as extrinsic, regulatory components involved in the water-splitting reaction. Among these three extrinsic proteins, the extrinsic 33 kDa protein is commonly found in PSII from higher plants to cyanobacteria with an overall sequence homology of 43−48% between higher plants and cyanobacteria (Kuwabara et al., 1987; Tyagi et al., 1987; Philbrick & Zilinskas, 1988). The other two extrinsic proteins of 23 and 17 kDa are present in higher plant PSII but are absent in cyanobacteria (Koike & Inoue, 1985; Stewart et al., 1985a; Shen et al., 1992). Instead, cyanobacterial PSII contains two additional extrinsic proteins, cytochrome (cyt) *c*-550 (Shen et al., 1992; Shen & Inoue, 1993a) and a 9−12 kDa protein (hereafter referred to as 12 kDa protein) (Stewart et al., 1985a,b; Shen et al., 1992; Shen & Inoue, 1993a), both of which are absent in higher plant PSII.

The function of the three extrinsic proteins in the oxygen-evolving reaction has been studied extensively mainly with isolated PSII membranes from higher plants [for a review, see Debus (1992)]. In higher plant PSII, the 33 kDa protein appears of crucial importance for functioning and stability of the Mn cluster; removal of the protein essentially eliminates oxygen evolution concomitant with a destabilization of the Mn binding unless a nonphysiologically high concentration of chloride was supplemented (Ono & Inoue, 1983, 1985; Miyao & Murata, 1984; Bricker, 1992). Moreover, genetic depletion of the 33 kDa protein in *Chlamydomonas reinhardtii* caused a complete loss of oxygen-evolving activity as well as a drastic decrease in the PSII abundance (Mayfield et al., 1987a). In cyanobacteria, however, depletion of the 33 kDa protein resulted in a mutant that can still grow photoautotrophically albeit with a 30−40% reduction in its growth rate (Burnap & Sherman, 1991;

[†] Supported by a grant on Photosynthetic Sciences provided to The Institute of Physical and Chemical Research (RIKEN) by The Science and Technology Agency (STA) of Japan and USDA-CRGO Grant 92-37506-7820 (to R.L.B.).

* Corresponding author. Fax: 81-48-462-4685. E-mail: shen@postman.riken.go.jp.

[‡] The Institute of Physical and Chemical Research (RIKEN).

[§] Oklahoma State University.

[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1995.

[1] Abbreviations: bp, base pair; chl, chlorophyll; CP43 and CP47, intrinsic chlorophyll-binding proteins associated with photosystem II reaction center; cyt, cytochrome; D1 and D2, reaction center proteins of photosystem II; PSII, photosystem II; TMBZ, 3,3′,5,5′-tetramethylbenzidine.

0006-2960/95/0434-12661$09.00/0

Bockholt et al., 1991; Mayes et al., 1991; Philbrick et al., 1991). In addition, the cyanobacterial mutant lacking the 33 kDa protein did not show a significant reduction in its PSII content (Burnap & Sherman, 1991; Philbrick et al., 1991; Burnap et al., 1992). These are apparently in contradiction with the results observed in the 33 kDa deletion mutant of *C. reinhardtii* and are also contrast with the role of the 33 kDa protein in plant PSII revealed by *in vitro* release-reconstitution studies. A possible cause underlying this difference is that cyanobacteria maintain a high concentration of chloride within their cells, which thus supports the functioning and stability of the Mn cluster in the absence of the 33 kDa protein. This idea, however, has not been substantiated so far.

Another possible cause for the difference observed upon deletion of the 33 kDa protein between higher plant PSII and cyanobacterial PSII may relate to the difference in the other two extrinsic proteins; as mentioned above, the higher plant PSII contains extrinsic 23 and 17 kDa proteins whereas cyanobacterial PSII contains cyt *c*-550 and a 12 kDa protein as extrinsic components. While both deletion mutants of the 23 kDa protein in *C. reinhardtii* and cyt *c*-550 in *Synechocystis* sp. PCC 6803 similarly showed a reduction in oxygen-evolving activity and also a destabilization in the PSII complex (Mayfield et al., 1987b; Shen et al., 1995), there is an apparent difference between the 23 kDa protein in higher plants and cyt *c*-550 in cyanobacteria, as the 23 kDa protein cannot bind to and function in the higher plant PSII in the absence of the 33 kDa protein (Miyao & Murata, 1987), but cyt *c*-550 alone can effectively bind to cyanobacterial PSII in the absence of the 33 kDa protein (Shen & Inoue, 1993a). This raises a possibility that, in cyanobacterial PSII, cyt *c*-550 alone may function in the absence of the 33 kDa protein and thus supports the oxygen evolution observed in the 33 kDa protein deletion mutant. In order to clarify this question and also explore the possible interaction between cyt *c*-550 and the 33 kDa protein in cyanobacterial PSII, we have made a double-deletion mutant lacking both the 33 kDa protein and cyt *c*-550 by simultaneously inactivating their genes, the *psb*O and *psb*V genes, in *Synechocystis* sp. PCC 6803, and analyzed its ability for photoautotrophic growth and PSII-related activities. It was revealed that the simultaneous loss of the 33 kDa protein and cyt *c*-550 yielded an obligatory photoheterotrophic phenotype in *Synechocystis* sp. PCC 6803 and that this loss of photoautotrophic growth was caused by the loss of PSII oxygen-evolving activity accompanied by destabilization of the PSII complex *in vivo*. These results clearly indicate that cyt *c*-550 can indeed function in the absence of the 33 kDa protein in cyanobacteria and that the oxygen evolution observed in the 33 kDa protein deletion mutant is supported by cyt *c*-550.

MATERIALS AND METHODS

Synechocystis sp. PCC 6803 was grown in BG11 at 30 °C at a light intensity of 30 μEinstein m^{-2} s^{-1} provided by white fluorescent tubes. A *psb*O$^-$ mutant, in which the *psb*O gene encoding the PSII extrinsic 33 kDa protein had been replaced by a spectinomycin-resistant cassette, was constructed as described by Burnap and Sherman (1991). This mutant was used as a host strain to construct the double-deletion mutant lacking both the 33 kDa protein and cyt *c*-550. To do so, the *psb*V gene encoding cyt *c*-550 was cloned into plasmid pUC119 and inactivated by inserting a kanamycin-resistant cassette into the *Hinc*II site 80 bp downstream from the translation start codon (Shen et al., 1995). The resultant plasmid, pCK550, was used to transform the *psb*O$^-$ mutant. The resultant transformant was selected several times on BG11 plates containing 20 μg of spectinomycin/mL, 5 μg of kanamycin/mL, 20 μM atrazine, and 5 mM glucose, and the resultant spectinomycin- and kanamycin-resistant mutant was designated as *psb*O$^-$/*psb*V$^-$.

To determine growth rate, cells of wild-type and mutant strains were inoculated in liquid BG11 medium either in the presence or in the absence of 5 mM glucose at 30 °C. The cell density was determined by measuring light scattering of the culture at 730 nm. For other characterization experiments, cells of wild-type strain were grown in BG11 while the mutant cells were grown in BG11 supplemented with 5 mM glucose; both wild-type and mutant cells were harvested in their midlogarithmic growth phase. The amount of PSII reaction center in intact cells was determined by measuring the binding capacity of ^{14}C-labeled atrazine to PSII according to Vermaas et al. (1990). Cells of wild-type or mutant strains were incubated with 100−1000 nM [^{14}C]-atrazine (specific activity, 25 mCi/mmol; Sigma) for 30 min at room temperature in the growth medium containing 2% ethanol at 50 μg of chl/mL, followed by a brief centrifugation to pellet the cells. The radioactivity in the supernatants was counted by a liquid scintillation counter, and the amount of bound [^{14}C]atrazine was calculated. For each concentration of [^{14}C]atrazine, duplicate samples were measured, and the obtained values were averaged. Nonspecific binding of [^{14}C]-atrazine to cells was determined in the presence of 20 μM 3,-(3,4-dichlorophenyl)-1,1-dimethylurea.

PSII oxygen-evolving activity was measured in BG11 medium at 30 °C with a Clark-type oxygen electrode under saturating light, with 0.5 mM 2,6-dichloro-*p*-benzoquinone and 1 mM potassium ferricyanide as electron acceptors at 10 μg of chl/mL. The whole-chain electron transport activity was measured in the presence of 10 mM $NaHCO_3$. For measuring thermoluminescence emission, the cells of wild-type and mutants were dark-adapted at room temperature for 10 min at 100 μg of chl/mL in the growth medium, illuminated with one, two, or three flashes and then immediately plunged into liquid nitrogen to cool the sample. Thermoluminescence glow curves were recorded as described by Ono and Inoue (1986), with a heating rate of 1 °C/s.

For electrophoresis, harvested cells were disrupted by ultrasonication as described previously (Shen & Inoue, 1993b; Shen et al., 1995). Polyacrylamide gel electrophoresis was carried out with a 16−22% gradient gel containing 6 M urea at room temperature (Ikeuchi & Inoue, 1988). For heme staining, the gel was first incubated with 3,3′,5,5′-tetramethylbenzidine (TMBZ), followed by an addition of H_2O_2 to develop the color (Thomas et al., 1976; Shen & Inoue, 1993b). For immunoblotting, proteins on the gel were transferred to a nitrocellulose membrane (Schleicher & Schuell, pore size 0.2 μm), incubated with respective antibodies, and visualized by alkaline phosphatase-conjugated goat antirabbit IgG (Jackson ImmunoResearch Laboratories, Inc.). An antibody against the extrinsic 12 kDa protein was raised against the protein purified from *Synechococcus vulcanus* (Shen & Inoue, 1993b); antibodies against the PSII D1, D2, CP47, CP43, and 33 kDa proteins were raised against respective spinach proteins by Dr. M. Ikeuchi.

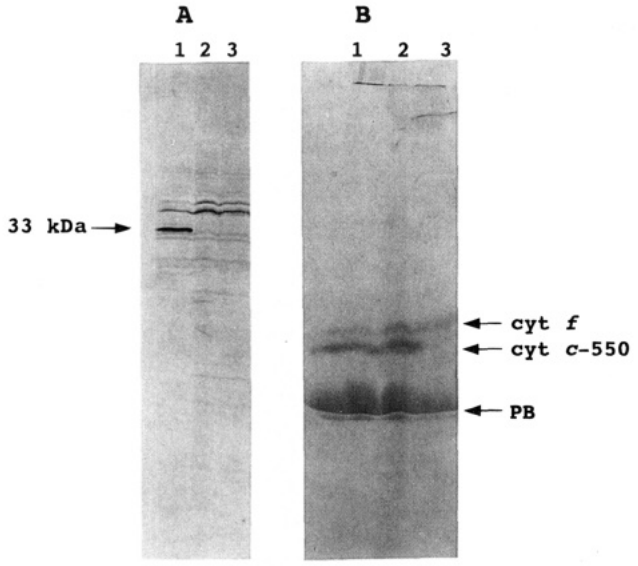

FIGURE 1: Immunoblot analysis with an antibody against the 33 kDa protein (A) and heme-staining (B) of whole-cell extracts of *Synechocystis* sp. PCC 6803 wild-type and mutant strains. Cells were disrupted by sonication as described in Materials and Methods, and the whole-cell extracts equivalent to 3 (A) or 15 μg of chlorophyll (B) were loaded in each lane: (lane 1) wild-type strain; (lane 2) *psb*O$^-$ mutant; (lane 3) *psb*O$^-$/*psb*V$^-$ mutant. PB, phycobiliproteins.

RESULTS

The *psb*O$^-$ mutant used in this study as a host strain to construct the double-deletion mutant was constructed and characterized previously (Burnap & Sherman, 1991). To construct the double-deletion mutant lacking both 33 kDa protein and cyt *c*-550, the *psb*O$^-$ host strain was transformed with the plasmid pCK550 that contains the *psb*V gene disrupted by a kanamycin-resistant cassette. After 2−3 months selection in the presence of both spectinomycin and kanamycin, mutants resistant to both antibiotics were obtained. Two strains were selected and analyzed for their growth, photosynthetic properties, and protein composition, and the obtained results were identical for the two strains. In the following, only the results for one of the two strains were presented; this strain was designated as *psb*O$^-$/*psb*V$^-$.

Replacement of the *psb*V gene with plasmid pCK550 in the double-deletion mutant was confirmed by PCR amplification of genomic DNA with primers flanking the *psb*V gene. While PCR amplification of genomic DNA from wild-type or *psb*O$^-$ strain yielded a fragment of 0.86 kb as expected (Shen et al., 1995), PCR amplification using genomic DNA from the *psb*O$^-$/*psb*V$^-$ strain gave rise to a fragment of 2.1 kb (data not shown), indicating that the *psb*V gene was successfully inactivated by homologous recombination with the segment of plasmid pCK550 in which the *psb*V gene has been interrupted by insertion of the kanamycin-resistant cassette of 1.3 kb (Shen et al., 1995).

In order to confirm the absence of both the 33 kDa protein and cyt *c*-550 in the *psb*O$^-$/*psb*V$^-$ strain, cells of wild-type and mutant strain were disrupted, electrophoresed, and analyzed either by immunoblotting with an antibody against the spinach 33 kDa protein or by heme staining with TMBZ/H_2O_2. For comparison, cell extracts from the *psb*O$^-$ strain were also analyzed. Immunoblotting with antibody against the 33 kDa protein produced one dense and one fainter band in the wild type cells (Figure 1A); the dense band is from cross-reaction of the antibody with the true 33 kDa protein whereas the fainter band is most likely due to some nonspecific cross-reaction of the antibody with a protein with some higher molecular weight, since the antibody we used was raised against the spinach 33 kDa protein. In the 33 kDa protein-less and double-deletion mutants, the nonspecific band became denser, probably because the amount of the antibody available for nonspecific cross-reaction was larger in these mutants since they lacked the 33 kDa protein. Regardless of the presence of this nonspecific band, the results obtained clearly show that (Figure 1), while the wild-type strain contained both the 33 kDa protein and cyt *c*-550 and the *psb*O$^-$ strain lacked the 33 kDa protein but retained cyt *c*-550, both the 33 kDa protein and cyt *c*-550 were completely absent in the *psb*O$^-$/*psb*V$^-$ strain, indicating successful construction of the double-deletion mutant.

Table 1: Comparison of Growth Rates, PSII Contents, and Photosynthetic Electron Transport Activities of *Synechocystis* sp. PCC 6803 Wild-Type (WT), *psb*O$^-$, and *psb*O$^-$/*psb*V$^-$ Mutant Strains[a]

	WT	*psb*O$^-$	*psb*O$^-$/*psb*V$^-$
doubling time in BG11 (h)			
−glucose	13.0	26.0	ng
+glucose	9.2	9.0	9.5
chlorophyll/PSII ratio[b]	650	670	3300
(relative)	(100)	(91)	(20)
electron transfer rates[c]			
whole chain, H_2O to $NaHCO_3$	125	75	13
(relative)	(100)	(60)	(10)
PSII, H_2O to DCBQ/FeCN	480	145	40
(relative)	(100)	(30)	(8)

[a] Numbers in parentheses are percentages relative to the wild-type strain. Abbreviations used in the table are ng, no growth, DCBQ, 2,6-dichloro-*p*-benzoquinone, and FeCN, potassium ferricyanide. [b] Determined with the herbicide-binding method using ^{14}C-labeled atrazine. [c] Expressed as micromoles of O_2 per milligram of chl per hour.

Figure 2 shows the growth curves of wild-type, *psb*O$^-$, and *psb*O$^-$/*psb*V$^-$ mutant strains either in the absence or in the presence of 5 mM glucose. In the presence of 5 mM glucose in the growth medium, no significant difference was observed between the growth rates of wild-type, the *psb*O$^-$ strain, and the *psb*O$^-$/*psb*V$^-$ strain, suggesting that the simultaneous loss of 33 kDa protein and cyt *c*-550 did not affect the photomixotrophic and photoheterotrophic growth of the cyanobacterium. In the absence of glucose, however, drastic effects were observed. While the growth rate of *psb*O$^-$ strain was about 60% (Table 1) of the wild-type strain in agreement with previous reports (Burnap & Sherman, 1991; Bockholt et al., 1991; Mayes et al., 1991; Philbrick et al., 1991), the *psb*O$^-$/*psb*V$^-$ strain could not grow at all in the absence of glucose. This indicates that the additional loss of cyt *c*-550 in the *psb*O$^-$ strain affected the cyanobacterium significantly, which in turn implies that cyt *c*-550 is able to function in supporting photoautotrophic growth even in the absence of the 33 kDa protein.

The loss of photoautotrophic growth in the *psb*O$^-$/*psb*V$^-$ mutant was correlated with a loss in the activity of the whole-chain electron transport determined with $NaHCO_3$ as an electron acceptor. As Table 1 shows, the *psb*O$^-$/*psb*V$^-$ strain showed virtually no activity of whole-chain electron transport with $NaHCO_3$ as acceptor, while the *psb*O$^-$ strain still retained 60% activity of whole-chain electron transport as compared with the wild-type strain, in agreement with previous reports (Burnap & Sherman, 1991). A similar

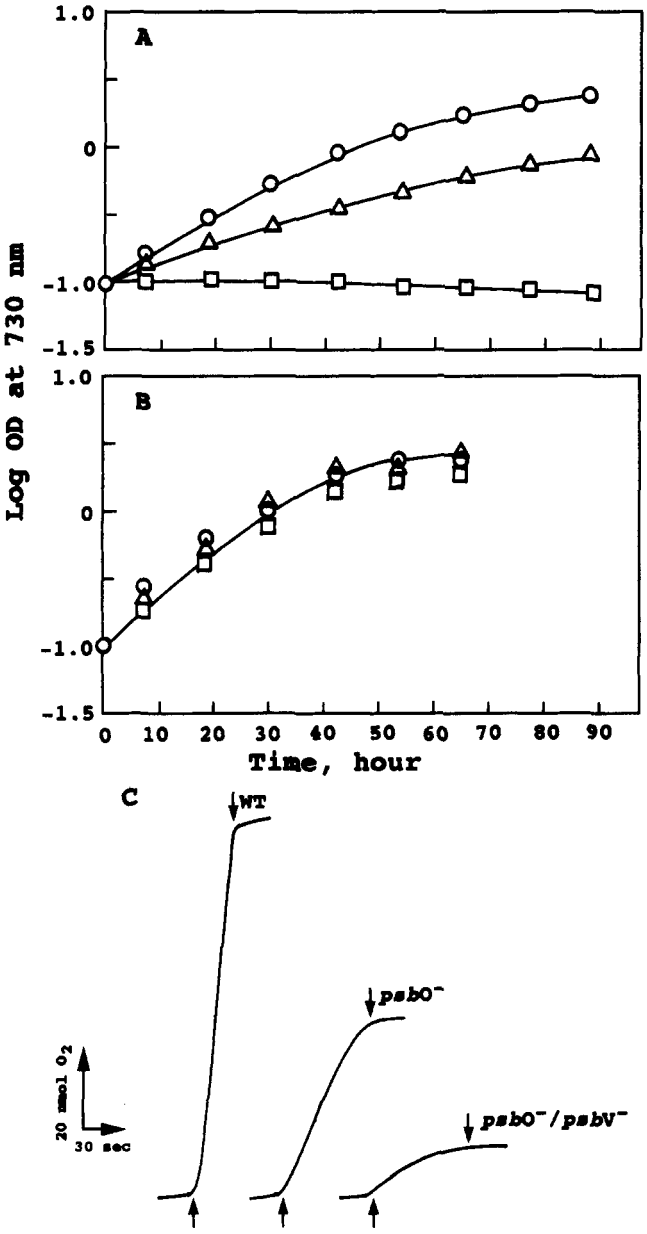

FIGURE 2: (A) Growth rate of *Synechocystis* sp. PCC 6803 wild-type and mutant strains in the absence of glucose: (○) wild-type strain; (△) *psb*O⁻ mutant; (□) *psb*O⁻/*psb*V⁻ mutant. (B) Growth rate of *Synechocystis* sp. PCC 6803 wild-type and mutant strains in the presence of 5 mM glucose. Symbols are the same as in (A). (C) Traces of PSII oxygen evolution from cells of *Synechocystis* sp. PCC 6803 wild-type and mutant strains determined polarographically with a Clark-type oxygen electrode at 30 °C under saturating light at 10 μg of chlorophyll/mL, with 0.5 mM 2,6-dichloro-*p*-benzoquinone and 1 mM potassium ferricyanide as acceptors.

situation was observed in PSII oxygen-evolving activity determined with 2,6-dichloro-*p*-benzoquinone and potassium ferricyanide as acceptors: the activity of *psb*O⁻ strain was 30% whereas that of the *psb*O⁻/*psb*V⁻ strain was only 8% of that observed in the wild-type strain. Although a trace of PSII activity was still detected for the mutant *psb*O⁻/*psb*V⁻, it has to be pointed out that this activity is close to the detection limit of our polarographic measurement. Moreover, the trace activity of PSII oxygen evolution observed in the double-deletion mutant disappeared rapidly upon illumination during measurement (Figure 2C), whereas such rapid loss of activity was not observed in the wild-

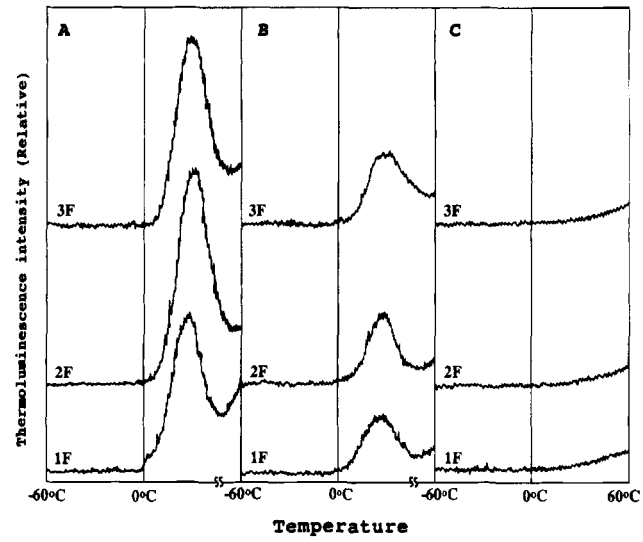

FIGURE 3: Thermoluminescence glow curves of *Synechocystis* sp. PCC 6803 wild-type and mutant cells. Cells were dark adapted for 10 min at room temperature at a chlorophyll concentration of 0.1 mg/mL, illuminated with one, two, or three flashes at 0 °C, and then immediately cooled to liquid nitrogen temperature. The thermoluminescence glow curves were recorded with a heating rate of 1 °C/s: (A) wild-type strain; (B) *psb*O⁻ mutant strain; (C) *psb*O⁻/*psb*V⁻ mutant strain.

type or *psb*O⁻ strain, although the *psb*O⁻ mutant did show somewhat fast damping of the activity upon illumination, as has been reported previously (Mayes et al., 1991; Philbrick et al., 1991). These results indicate that the additional loss of cyt *c*-550 in the *psb*O⁻ mutant gives rise to a significant influence on the PSII oxygen-evolving complex, which then leads to the loss of the ability of photoautotrophic growth in the double-deletion mutant, *psb*O⁻/*psb*V⁻. In addition, the rapid loss of the residual trace activity during measurement implies that the PSII complex in the mutant cells is more sensitive to photoinhibition, which is a typical phenomenon observed for PSII having impairments at its donor side.

Thermoluminescence emission is a good means to monitor the state of the oxygen-evolving complex and the PSII acceptor side. When dark-adapted cells are given one, two, or more flashes, the oxygen-evolving complex goes to S_2, S_3, or subsequent S states with parallel accumulation of negative charges on the Q_B site in the absence of herbicide. The separated charges can be stabilized by cooling the sample immediately following flash illumination, and these positive and negative charges will recombine with each other at characteristic temperatures upon warming the sample; as a result of charge recombination, weak light is emitted [for a review, see Vass and Inoue (1992)]. In order to further characterize the PSII oxygen-evolving complex in the double-deletion mutant, we measured thermoluminescence emission from cells of wild-type and mutant strains. As Figure 3 shows, dark-adapted wild-type cells showed significant light emission after the first flash and the intensity of emission increased remarkably after the second flash but decreased after the third flash. The stronger emission after two flashes as compared with that after one flash may reflect the situation that the dominant initial charge pair in the cells is $S_1Q_B^-$ after a short period of dark adaptation, which would result in limitation in the negative charge in the Q_B site after one flash due to formation of Q_B^{2-} and its subsequent easy release from the binding site (Velthuys, 1981; Wraight, 1981).

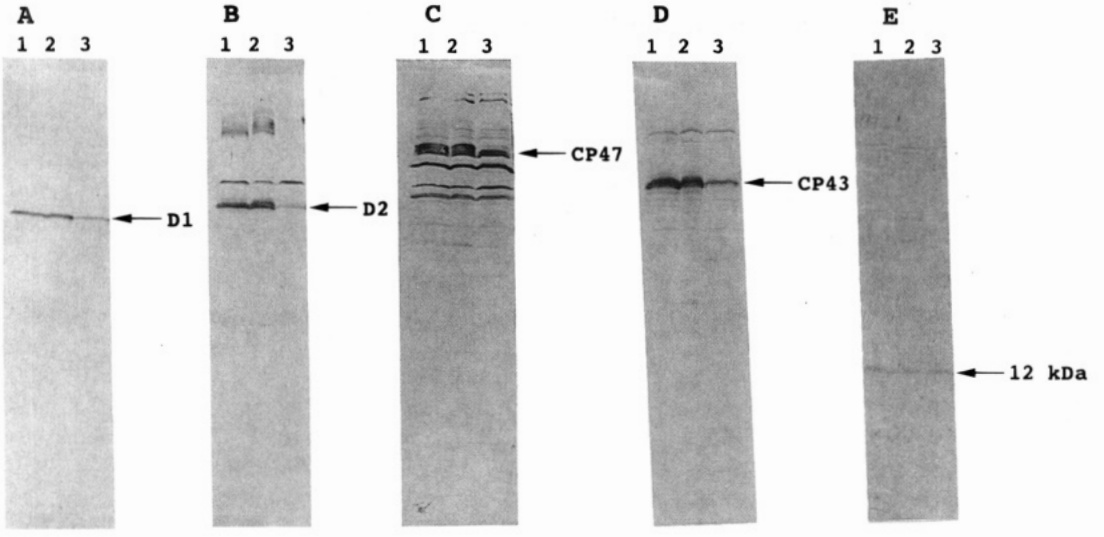

FIGURE 4: Immunoblot analysis of whole-cell extracts of *Synechocystis* sp. PCC 6803 wild-type and mutant strains with antibodies against D1 (A), D2 (B), CP47 (C), CP43 (D), and extrinsic 12 kDa protein (E). Cells were disrupted by sonication, and whole-cell extracts equivalent to 3 μg of chlorophyll were loaded in each lane: (lane 1) wild-type strain; (lane 2) *psb*O$^-$ mutant strain; (lane 3) *psb*O$^-$/*psb*V$^-$ mutant strain.

Alternatively, giving that the initial state is S_1Q_B after dark adaptation, the $S_3Q_B^-$ charge pair formed after two flashes may emit thermoluminescence much more strongly than the $S_2Q_B^-$ charge pair formed after one flash upon recombination in cyanobacterial PSII, although in higher plant PSII, the thermoluminescence yield of the $S_3Q_B^-$ charge pair has been determined to be 1.7−2.0-fold that of the $S_2Q_B^-$ charge pair (Rutherford et al., 1984; Demeter et al., 1985). In any case, the obtained thermoluminescence emission pattern is the same as we previously reported for cyanobacterial cells and indicates a normal S-state transition in the wild-type cells (Shen & Inoue, 1993a; Burnap et al., 1992). Cells of the *psb*O$^-$ mutant emitted thermoluminescence at a reduced intensity that was comparable to its oxygen-evolving activity, although its oscillation pattern was somewhat changed with the largest emission appeared after the third flash [see also Burnap et al. (1992) and Vass et al. (1992)]. Cells of the double-deletion mutant, however, showed virtually no thermoluminescence emission either after one, two, or three flashes. This appears somewhat different from what would be expected from the oxygen-evolving activity measured under continuous illumination for the mutant cells, since a trace activity was still detected for the double-deletion mutant. This apparent discrepancy probably results from a rapid dark deactivation of the oxygen-evolving complex that occurred during the period of dark adaptation prior to thermoluminescence measurement, since we have observed a similar rapid dark deactivation in the cyt *c*-550-deletion mutant (data to be published). Regardless of this possible rapid dark deactivation, the lack of thermoluminescence emission in the double-deletion mutant unambiguously indicates that the PSII oxygen-evolving complex devoid of both the 33 kDa protein and cyt *c*-550 is essentially incapable of undergoing normal S-state transitions. Since the mutant lacking the 33 kDa protein alone is still capable of S-state transitions albeit with a reduced rate (Burnap et al., 1992; Vass et al., 1992), this again indicates that inactivation of the oxygen-evolving complex in the double-deletion mutant is caused by the further deletion of cyt *c*-550.

As we have reported previously (Shen et al., 1995), genetic inactivation of the *psb*V gene coding for cyt *c*-550 not only reduced the PSII oxygen-evolving activity but also destabilized the PSII reaction center complex, yielding mutants with reduced PSII contents (53-67%) as compared with the wild-type strain. In order to examine whether similar or severer effects occurred upon simultaneous depletion of the 33 kDa protein and cyt *c*-550, we measured the PSII content in cells of wild-type and mutant strains by determining the ratio of chl to PSII with the herbicide-binding method using ^{14}C-labeled atrazine. The K_D values obtained for [^{14}C]atrazine were 120−250 nM; these are comparable with the values reported by Tommos et al. (1993) and Shen et al. (1995). The ratio determined for the wild-type strain was 650 chlorophylls/binding site of atrazine (Table 1), similar to the values previously obtained for this cyanobacterium (Vermaas et al., 1990; Burnap et al., 1992; Tommos et al., 1993; Shen et al., 1995). A slightly lower PSII abundance was obtained for the mutant *psb*O$^-$ under our growth condition, indicating that loss of the 33 kDa protein had, if any, only a minor effect on the stability of PSII *in vivo*, consistent with the results obtained previously (Burnap & Sherman, 1991; Philbrick et al., 1991; Burnap et al., 1992). The chl to PSII ratio, however, increased drastically in the double-deletion mutant, *psb*O$^-$/*psb*V$^-$, resulting in a PSII abundance as low as 20% in the mutant as compared with that in the wild-type strain. This value is much lower than that observed in the single-deletion mutant lacking cyt *c*-550 (53−67%) (Shen et al., 1995), thus indicating a much increased instability of the PSII complex in the double-deletion mutant.

The PSII abundance obtained for the double-deletion mutant was so low that it was close to the detection limit of the herbicide-binding method. To examine more precisely the effects of simultaneous loss of 33 kDa protein and cyt *c*-550 on the stability of PSII complex, we further determined the contents of major PSII subunits D1, D2, CP47, and CP43 as well as the extrinsic 12 kDa protein by means of immunoblot analysis with antibodies raised against these respective polypeptides. As Figure 4 shows, cells of wild type and the *psb*O$^-$ mutant showed similar band intensities of Western blot for all the polypeptides detected, indicating again that loss of the 33 kDa protein alone gave no significant effect on the PSII stability, in agreement with results reported

previously and also with the above herbicide-binding experiments. In the double-deletion mutant, a drastic decrease in the intensity of Western blotting was observed with antibodies against PSII intrinsic proteins D1, D2, CP47, and CP43. This decrease is qualitatively similar to those found in the PSII abundance determined with the herbicide-binding method and thus reinforces the conclusion derived from herbicide-binding experiments that simultaneous loss of the 33 kDa protein and cyt *c*-550 remarkably destabilizes the PSII complex *in vivo*. Western blot with an antibody against the extrinsic 12 kDa protein of PSII from thermophilic *Synechococcus vulcanus* also detected a polypeptide of similar molecular weight in *Synechocystis* 6803, a mesophilic cyanobacterium, confirming that the 12 kDa extrinsic protein is commonly present in both thermophilic and mesophilic cyanobacteria. The intensity of this band, however, did not much differ between the wild-type, *psb*O$^-$ and *psb*O$^-$/*psb*V$^-$ strains. This implies that the 12 kDa protein is stable even in the absence of the PSII reaction center complex. Similar situations have been observed for the 33 kDa extrinsic protein in cyanobacterial mutants with destabilized PSII complex (Nilsson et al., 1990; Ikeuchi et al., 1992; Mor et al., 1993; Gleiter et al., 1994).

DISCUSSION

Functional Relationships between the 33 kDa Protein and Cyt c-550 in Cyanobacterial PSII. The present study showed that simultaneous deletion of the PSII extrinsic 33 kDa protein and cyt *c*-550 virtually inactivated the PSII oxygen-evolving complex in the cyanobacterium *Synechocystis* sp. PCC 6803, resulting in a mutant that can evolve oxygen at a rate of only 8% of that in the wild-type strain. Moreover, the PSII oxygen-evolving complex in this mutant appears to be more sensitive to photoinhibition, as the activity was lost rapidly under illumination; this is typical for PSII with a damaged donor side. Thermoluminescence measurement indicated that virtually no detectable charge accumulation occurred in the double-deletion mutant. Although this seems to contradict the faint but detectable oxygen evolution under continuous illumination, it is likely that the complete lack of thermoluminescence emission is due to a fast dark deactivation of the PSII oxygen-evolving complex in the double-deletion mutant, since a similar fast dark deactivation has been observed in the single-deletion mutant lacking cyt *c*-550 (data to be published). All the present observations thus indicate that the PSII oxygen-evolving complex in the double-deletion mutant is significantly damaged. As a result, this mutant cannot grow photoautotrophically. Since mutants lacking either the 33 kDa protein or cyt *c*-550 alone were still capable of oxygen evolution and photoautotrophic growth although with reduced rates (Burnap & Sherman, 1991; Bockholt et al., 1991; Mayes et al., 1991; Philbrick et al., 1991; Shen et al., 1995), the present results indicate that either of these two extrinsic proteins is capable of supporting oxygen evolution independently, in the absence of the other, although both of them are required for normal, maximal rate of oxygen evolution in cyanobacterial PSII. In other words, oxygen evolution and photoautotrophic growth in the *psb*O$^-$ mutant is supported by cyt *c*-550 and *vice versa*. This not only provides further evidence for the concept that cyt *c*-550 is an extrinsic component required for maintaining cyanobacterial oxygen evolution (Shen et al., 1992; Shen & Inoue, 1993a,b; Shen et al., 1995) but also presents a unique feature for its mode of function in cyanobacterial PSII.

Although both the 33 kDa protein and cyt *c*-550 are required for normal oxygen evolution and photoautotrophic growth in cyanobacteria, there exists an important functional difference between these two extrinsic components. Upon loss of the 33 kDa protein alone, no significant change in the PSII stability was observed [Burnap and Sherman (1991), Philbrick et al. (1991), Burnap et al. (1992), and this study], but loss of cyt *c*-550 decreased the PSII abundance to 53−67% of that in the wild-type strain (Shen et al., 1995). This difference is in line with the above conclusion that the 33 kDa protein and cyt *c*-550 can function independently of each other. Upon simultaneous loss of the 33 kDa protein and cyt *c*-550, however, the PSII content decreased to about 20% of that observed in the wild-type strain (Table 1, Figure 4). Apparently, the destabilizing effect caused by the loss of cyt *c*-550 became more pronounced in the absence of the 33 kDa protein. Probably, the inactive PSII reaction centers formed upon deletion of the 33 kDa protein became more structurally unstable upon further loss of cyt *c*-550 and thus are prone to rapid degradation.

Loss of PSII complex will obviously result in an apparent loss of oxygen-evolving activity. In fact, if we correct the observed activity in the double-deletion mutant by taking into account the loss of PSII reaction centers, the activity observed in the double-deletion mutant is 40% of that observed in the wild-type strain. This is comparable with the activity observed in the single-deletion mutant lacking the 33 kDa protein, thus implying that further loss of cyt *c*-550 in the 33 kDa protein-less mutant mainly destabilized the PSII complex. This is in line with our previous observations that deletion of cyt *c*-550 alone significantly destabilized the PSII complex but loss of the activity was rather small after correction for loss of PSII reaction centers (Shen et al., 1995). We must emphasize at this point, however, that it is difficult to determine whether loss of the activity *in vivo* is due to destabilization of PSII centers, or inversely, destabilization of PSII is a result of formation of inactive PSII complexes, since inactive PSII complexes are known to be degraded rapidly. From our previous *in vitro* reconstitution and *in vivo* deletion mutagenesis studies (Shen & Inoue, 1993a; Shen et al. 1995), we conclude that cyt *c*-550 also has some important regulatory function in the cyanobacterial oxygen-evolving reactions in addition to its role in maintaining PSII stability.

The finding that cyt *c*-550 functions independently of the 33 kDa protein *in vivo* in cyanobacterial cells seems to contradict the results of *in vitro* reconstitution experiments (Shen & Inoue, 1993a): In the *in vitro* reconstitution experiments, we have observed that although cyt *c*-550 can rebind to purified, $CaCl_2$-washed PSII in the absence of the 33 kDa protein, this binding did not give significant enhancement of the oxygen-evolving activity. Obvious effects of cyt *c*-550 rebinding were observed only when the 33 kDa protein was already bound, which suggested that cyt *c*-550 can function only in the presence of the 33 kDa protein. The cause for the apparent discrepancy between the *in vitro* reconstitution and *in vivo* deletion mutagenesis studies is not clear at present, but several possibilities should be considered: (1) The *in vitro* reconstitution experiments were done with $CaCl_2$-washed purified PSII. The $CaCl_2$ wash may have imposed additional structural damages that did not occur in

deletion mutagenesis. (2) Even though the $CaCl_2$ wash did not induce any additional damages, the purification procedures employed for preparation of the PSII complex may have eliminated some unknown factors required for functioning of cyt *c*-550 in the absence of the 33 kDa protein. (3) Cyt *c*-550 may acquire some additional function in cyanobacterial cells to replace partly the function of the 33 kDa protein in maintaining oxygen evolution when the 33 kDa protein had been taken out genetically. (4) The reconstitution experiments were done with PSII from a thermophilic cyanobacterium in which the mode of function of cyt *c*-550 may differ from that in the mesophilic cyanobacterium used in the *in vivo* deletion mutagenesis. (5) The function of cyt *c*-550 is mainly stabilization of the PSII complex so that its function cannot be manifested in the *in vitro* reconstitution experiments. [However, this is unlikely since reconstitution of cyt *c*-550 together with the 33 kDa protein apparently enhanced oxygen evolution [Shen & Inoue 1993a)]. At present, we cannot distinguish among these possibilities which is responsible for the apparent discrepancy between the results of *in vitro* reconstitution and *in vivo* deletion mutagenesis studies; a similar reconstitution study with PSII purified from *Synechocystis* sp. PCC 6803 should clarify this question.

Comparison of Extrinsic Proteins between PSII of Higher Plants and Cyanobacteria. Cyt *c*-550, together with the 12 kDa protein, presents a major difference between PSII of higher plants and cyanobacteria since, as mentioned in the introduction, higher plant PSII contains the 23 and 17 kDa extrinsic proteins instead of cyt *c*-550 and the 12 kDa protein. While the 12 kDa protein in cyanobacteria and 17 kDa protein in higher plants are similar in terms of their binding to PSII, i.e., neither protein can functionally bind to PSII in the absence of the other two extrinsic proteins (Miyao & Murata; 1987; Shen & Inoue, 1993a), interesting similarities and differences between cyt *c*-550 and the 23 kDa protein can be pointed out. One functional similarity between cyt *c*-550 and the 23 kDa protein is that the absence of either protein *in vivo* causes a reduction in oxygen evolution as well as a destabilization of PSII complex (Mayfield et al., 1987b; Shen et al., 1995). On the other hand, the previous and present results reveal an important difference, since the 23 kDa protein cannot bind and function in the absence of the 33 kDa protein (Miyao & Murata, 1987) but cyt *c*-550 alone can effectively bind and function without the 33 kDa protein (Shen & Inoue, 1993a and this study). This difference may account for the observed difference between the 33 kDa protein deletion mutant of *C. reinhardtii* and *Synechocystis* sp. PCC 6803. As has been reported, the 33 kDa protein deletion mutant of *C. reinhardtii* showed essentially no oxygen evolution and also a drastic decrease in the PSII content (Mayfield et al., 1987a), but cyanobacterial mutants lacking the 33 kDa protein were still capable of oxygen evolution and also showed no significant decrease in the PSII content (Burnap & Sherman, 1991; Mayes et al., 1991; Philbrick et al., 1991; Burnap et al., 1992). Since the 33 kDa proteins from higher plants and cyanobacteria have significant homology in their sequences, it is reasonable to assume that the function of this protein is the same between higher plants and cyanobacteria. Consequently, the difference observed between *C. reinhardtii* mutant and cyanobacterial mutant lacking the 33 kDa protein would be due to the difference in the functionality of the two extrinsic proteins attached: The 23 kDa protein will no longer be functional in the 33 kDa protein deletion mutant of *C. reinhardtii*, whereas cyt *c*-550 is still functional in the 33 kDa protein deletion mutant of cyanobacteria.

In conclusion, like the 33 kDa protein, cyt *c*-550 is an extrinsic component that can also bind and function independently in cyanobacterial PSII to support oxygen evolution and photoautotrophic growth. This not only provides this cyanobacterial cyt with a unique characteristic that is not found for the 23 kDa extrinsic protein in higher plant PSII but also accounts for the difference observed in the phenotype of mutants lacking the 33 kDa protein between *C. reinhardtii* and cyanobacteria.

ACKNOWLEDGMENT

We thank Dr. M. Ikeuchi for providing antibodies against CP47, CP43, D1, D2, and the 33 kDa protein.

REFERENCES

Bockholt, R., Masepohl, B., & Pistorius, E. K. (1991) *FEBS Lett. 294,* 59−63.
Bricker, T. (1992) *Biochemistry 31,* 4623−4628.
Burnap, R. L., & Sherman, L. (1991) *Biochemistry 30,* 440−446.
Burnap, R. L., Shen, J.-R., Jursinic, P. A., Qian, M., Inoue, Y., & Sherman, L. A. (1992) *Biochemistry 31,* 7404−7410.
Debus, R. J. (1992) *Biochim. Biophys. Acta 1102,* 269−353.
Demeter, S., Rosza, Zs, Vass, I., & Hideg, E. (1985) *Biochim. Biophys. Acta 809,* 379−387.
Erickson, J. M., & Rochaix, J.-D. (1992) in *Topics in Photosynthesis. The Photosystems: Structure, Function and Molecular Biology* Barber, J., Ed. Vol. 11, pp 101−177 Elsevier, Amsterdam, The Netherlands.
Gleiter, H. M., Haag, E., Shen, J.-R., Eaton-Rye, J. J., Inoue, Y., Vermaas, W. F.J., & Renger, G. (1994) *Biochemistry 33,* 12063−12071.
Ikeuchi, M. (1992) *Bot. Mag. Tokyo 105,* 327−373.
Ikeuchi, M., & Inoue, Y. (1988) *Plant Cell Physiol. 29,* 1233−1239.
Ikeuchi, M., Shen, J.-R., Vermaas, W. F. J., Pakrasi, H. B., Diner, B. A., Nixon, P.J., Kuriyama, A., Hirano, A., & Inoue, Y. (1992) in *Reserach in Photosynthesis*, Murata, N., Ed. Vol. II, pp 175−178, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Koike, H., & Inoue, Y. (1985) *Biochim. Biophys. Acta 807,* 64−73.
Kuwabara, T., Teddy, K. J., & Sherman, L. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84,* 8230−8234.
Mayes, S. R., Cook, K. M., Self, S. J., Zhang, Z., & Baber, J. (1991) *Biochim. Biophys. Acta 1060,* 1−12.
Mayfield, S. P., Bennoun, P., & Rochaix, J.-D. (1987a) *EMBO J. 6,* 313−318.
Mayfield, S. P., Rahire, M., Frank, G., Zuber, H., & Rochaix, J.-D. (1987b) *Pro. Natl. Acad. Sci. U.S.A. 84,* 749−753.
Miyao, M., & Murata, N. (1984) *FEBS Lett. 170,* 350−354.
Miyao, M., & Murata, N. (1987) in *Topics in Photosynthesis: Photoinhibition* (Kyle, D. J., Osmond, C. B., & Arntzen, C. J., Eds.) Vol. 9, pp 289−307, Elsevier, Amsterdam.
Mor, T. S., Post, A. E., & Ohad, I. (1993) *Biochim. Biophys. Acta 1141,* 206−212.
Nilsson, F., Andersson, B., & Jansson, C. (1990) *Plant Mol. Biol. 14,* 1051−1054.
Ono, T., & Inoue, Y. (1983) *FEBS Lett. 164,* 255−260.
Ono, T., & Inoue, Y. (1985) *Biochim. Biophys. Acta 806,* 331−334.
Ono, T., & Inoue, Y. (1986) *Biochim. Biophys. Acta 850,* 380−389.
Philbrick, J. B., & Zilinskas, B. A. (1988) *Mol., Gen. Genet. 212,* 418−425.
Philbrick, J. B., Diner, B. A., & Zilinskas, B. A. (1991) *J. Biol. Chem. 266,* 13370−13376.
Rutherford, A. W., Renger, G., Koike, H., & Inoue, Y. (1984) *Biochim. Biophys. Acta 767,* 548−556.

Shen, J.-R., & Inoue, Y. (1993a) *Biochemistry, 32,* 1825−1832.
Shen, J.-R., & Inoue, Y. (1993b) *J. Biol. Chem. 268,* 20408−20413.
Shen, J.-R., Ikeuchi, M., & Inoue, Y (1992) *FEBS Lett. 301,* 145−149.
Shen, J. -R., Vermaas, W., & Inoue, Y. (1995) *J. Biol. Chem. 270,* 6901−6907.
Stewart, A. C., Ljungberg, U., Åkerlund, H.-E., & Andersson, B. (1985a) *Biochim. Biophys. Acta 808,* 353−362.
Stewart, A. C., Siczkowski, M., & Ljungberg, U. (1985b) *FEBS Lett. 193,* 175−179.
Thomas, P. E., Ryan, D., & Levin, W. (1976) *Anal. Biochem. 75,* 168−176.
Tommos, C., Davidsson, L., Svensson, B., Madsen, C., Vermaas, W., & Styring, S. (1993) *Biochemistry, 32,* 5436−5441.
Tyagi, A., Hermans, J., Steppuhn, J., Jansson, C., Vater, F., & Herrmann, R. G. (1987) *Mol., Gen. Genet. 207,* 288−293.
Vass, I., & Inoue, Y. (1992) in *Current Topics in Photosynthesis: The Photosystems: Structure, Function and Molecular Biology* Barber, J., Ed., Vol. 11, pp 259−294, Elsevier, Amsterdam, The Netherlands.
Vass, I., Cook, K. M., Deak, Z., Mayes, S. M., & Baber, J. (1992) *Biochim. Biophys. Acta 1102,* 195−201.
Velthuys, B. R. (1981) *FEBS Lett. 126,* 277−281.
Vermaas, W., Charite, J., & Shen, G. (1990) *Z. Naturforsch. 45C,* 359−365.
Wraight, C. A. (1981) *Israel J. Chem. 21,* 348−354.

BI951097I