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Registry No. O_2, 7782-44-7; cytochrome *c* oxidase, 9001-16-5.

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Deletion Mutagenesis in *Synechocystis* sp. PCC6803 Indicates That the Mn-Stabilizing Protein of Photosystem II Is Not Essential for O_2 Evolution†

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ABSTRACT: The photosystem II (PSII) reaction center complex coordinates a cluster of Mn atoms that are involved in the accumulation of oxidizing equivalents generated by light-induced charge separations within the intrinsic portion of the PSII complex. A 33-kDa extrinsic protein, termed the Mn-stabilizing protein (MSP), has been implicated in the stabilization of two of the four Mn atoms of the cluster, yet the precise role of this protein in O_2 evolution remains to be elucidated. Here we describe the construction of a mutant of the cyanobacterium *Synechocystis* sp. PCC6803 in which the entire gene encoding MSP has been deleted. Northern and immunoblot analyses indicate that other PSII proteins are expressed and accumulated, despite the absence of MSP. Fluorescence emission spectra at 77 K indicate PSII assembles in the mutant, but that the binding of MSP is required for the normal fluorescence characteristics of the PSII complex, and suggest a specific interaction between MSP and CP47. Fluorescence induction measurements indicate a reduced rate of forward electron transport to the primary electron donor, P680, in the mutant. It is concluded that in contrast to previous reports, MSP is not required for the assembly of active PSII complexes nor is it essential for H_2O-splitting activity in vivo.

The photosystem II (PSII)[1] complex found in higher plants, eukaryotic algae, and cyanobacteria catalyzes the light-driven transport of electrons from H_2O to the mobile lipophilic electron carrier plastoquinone [for a review, see Babcock et al. (1989)]. The catalytic site of H_2O oxidation is known to contain a cluster of Mn atoms involved in the accumulation of oxidizing equivalents utilized for the four-electron oxidation of two molecules of H_2O. Biochemical resolution of the PSII complex has resulted in the identification of five intrinsic membrane proteins (CP47, CP43, D1, D2, and cytochrome b_{559}) which bind the pigments and cofactors involved in light capture and primary photochemistry. While the reaction center components mediating the initial charge separation and stabilization events are now thought to be located within a heterodimer formed by the D1 and D2 proteins, the proteins involved in binding the Mn cluster remain to be identified. The Mn cluster is coupled to the photooxidizable reaction center chlorophyll (P680) via an oxidizable tyrosyl residue (Y_z) of the D1 polypeptide (Debus et al., 1988; Metz et al., 1989). Portions of the D1/D2 heterodimer situated on the lumenal side of the thylakoid membrane are proposed to provide ligands for the Mn cluster (Babcock et al., 1989).

In addition to the membrane-spanning polypeptides, the PSII complex possesses one or more extrinsic polypeptides situated on the lumenal side of the thylakoid membrane in

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[1] Abbreviations: Chl, chlorophyll; CP47, 47-kDa PSII chlorophyll protein encoded by the *psbB* gene; cyt, b_{559}, cytochrome b_{559}, encoded by the *psbE/F* operon; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; D1 and D2, PSII reaction center proteins encoded by the *psbA* and *psbD* genes, respectively; FeCN, $K_3Fe(CN)_6$; F_{max}, maximum fluorescence yield, with all Q_A reduced; F_0, initial fluorescence yield, with all Q_A oxidized; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P680, chlorophyll molecule(s) which act(s) as the primary electron donor of the PSII reaction center; HQ, hydroquinone; MSP, manganese-stabilizing protein, extrinsic 33-kDa PSII protein encoded by the *psbO* gene; PSII, photosystem II.

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close proximity to the catalytic site of H_2O splitting (Homann, 1987). Higher plants and eukaryotic algae have three such polypeptides with approximate molecular weights of 33K, 23K, and 16K. Cyanobacteria possess the 33-kDa extrinsic protein but apparently lack homologues to the 23- and 16-kDa proteins. The precise function of the 33-kDa protein has been particularly difficult to ascertain. Genetic and developmental studies have led to the conclusion that the 33-kDa protein is required to O_2 evolution in vivo (Mayfield et al., 1987; Philbrick & Zilinskas, 1988; Mizobuchi & Yamamoto, 1989; de Vitry et al., 1989). Experiments using PSII particles isolated from spinach have established a direct relationship between the removal of the 33-kDa protein and the loss of two of the four active-site Mn (Kuwabara et al., 1985), raising the possibility that its amino acid side chains supply ligands to the Mn. These results have led to the designation of this protein as the Mn-stabilizing protein (MSP). Experiments testing the effect of MSP removal upon the S_2-state multiline ESR signal, a sensitive indicator of the ligand environment of the Mn cluster, have yielded conflicting results (Hunziker et al., 1987; Miller et al., 1987). However, it has also been shown that high concentrations of Cl^- stabilize the binding of otherwise labile Mn and restore significant levels of O_2 evolution following the Mn-preserving extraction of MSP (Kuwabara et al., 1985). These results indicate that if MSP indeed provides ligands to Mn, such ligands can undergo facile substitution. Alternatively, the binding of MSP to the intrinsic portion of the reaction center may stabilize a conformation of Mn binding domains present on other PSII polypeptides. In the present study, we analyze a mutant of *Synechocystis* in which the entire *psbO* gene has been deleted. In contrast to previous results, the MSP-less mutant forms stable PSII reaction centers and is capable of evolving O_2 at one-third the rate of the wild type.

Materials and Methods

The glucose-utilizing strain of *Synechocystis* sp. PCC6803 (henceforth referred to as the wild-type) and its derivatives were grown and manipulated as described previously (Williams, 1988), except that the BG-11 growth media was modified to contain twice the usual amount of $NaNO_3$. A strain of *Synechocystis* sp. PCC6803 in which the *psbE/F* operon is deleted was provided by Dr. H. Pakrasi (Washington University). Extraction of chromosomal DNA from *Synechocystis* sp. PCC6803 was performed essentially as described by Williams (1988). Standard recombinant DNA procedures were used for plasmid DNA isolation, restriction digests, isolation of DNA fragments, cloning, and propagation of *Escherichia coli* strains (Sambrook et al., 1989).

The *psbO* gene of *Synechocystis* sp. PCC6803 was isolated by probing a fragment-enriched library with the previously cloned *psbO* gene from *Synechococcus* PCC7942 (Kuwabara et al., 1987). Briefly, a 5.3-kb *Hin*dIII fragment was identified by Southern analysis using the *Synechococcus* PCC7942 *psbO* gene as a hybridization probe under conditions of reduced stringency. Double restriction enzyme digests further indicated that the *psbO* gene was contained in a 1.9-kb *Avr*II–*Hin*dIII fragment as expected based upon an independent analysis of the gene (Philbrick & Zilinskas, 1988). A gene library highly enriched in this fragment was constructed as follows: chromosomal DNA was digested with *Hin*dIII and separated on a low melting point agarose gel. DNA from the 5–6-kb region of the gel was purified, digested with *Avr*II, and subjected to a second round of preparative electrophoresis, this time selecting fragments in the 1.5–2.0-kb region of the gel. Cloning of the gel-purified *Avr*II/*Hin*dIII fragments took advantage of the compatibility of *Avr*II termini with *Xba*I termini, which, when ligated, forms a joint cut by neither enzyme. Accordingly, the plasmid Bluescript KS(−) was digested at the *Xba*I and *Hin*dIII sites within the polylinker and ligated to the mixture of purified *Avr*II/*Hin*dIII fragments. This strategy allowed the utilization of the *Xba*I site downstream of the *psbO* during subsequent mutagenesis procedures without having to resort to partial restriction enzymes digests. Following transformation into *E. coli* strain XL1-Blue and blue/white selection on X-Gal plates, colonies containing plasmids with inserts were gridded onto solid media for further screening. Colonies harboring the plasmid with the *psbO*-containing 1.9-kb *Avr*II–*Hin*dIII insert were identified essentially as described by Van der Plas et al. (1987). Plasmid DNA was extracted from several of the positives clones and subjected to restriction enzyme and dideoxy sequence analysis to confirm the presence of the *psbO* gene. One of the resultant plasmids, designated pRB1 (not shown), was used for further manipulation of the *psbO* gene.

To obtain a strain of *Synechocystis* in which the entire *psbO* gene is deleted at its chromosomal locus, a mutagenic plasmid was constructed by replacing the 1.37-kb *Afl*II–*Xba*I fragment of pRB1 containing the *psbO* coding sequence with the 2-kb Ω fragment encoding Sp^r and Sm^r, which had been excised from pRL463 (a gift from Dr. J. Elhai, Michigan State University). This plasmid, designated pRB42 (Figure 1, panel A), was then used to transform wild-type *Synechocystis* sp. PCC6803 to Sp^r utilizing established procedures (Williams, 1988) followed by photoheterotrophic growth on selective plates containing 5 mM glucose, 10 μM DCMU, and 5 μg/mL Sp.

PCR amplification of the *psbO* gene was performed according to published procedures (Sambrook et al., 1989) using *Taq* DNA polymerase and two 30 bp oligonucleotides (G+C contents of 54 and 56%) which flank the 822 bp *psbO* coding region. One hundred microliter reactions containing 1 μg of genomic DNA were subjected to a thermal cycling routine consisting of a 1-min denaturation step at 94 °C, a 1-min annealing step at 55 °C, and a chain elongation step for 1.5 min at 72 °C. After 40 iterations of the thermal cycle, 10 μL of the reaction products was analyzed on an ethidium bromide stained 1.2% agarose gel. The identity of the PCR product was verified by sequencing single-stranded template generated in a separate asymmetric amplification reaction (Sambrook et al., 1989).

RNA isolation and Northern analysis were performed according to Reddy et al. (1990). Plasmids containing *psbA*-2 and *psbD/C* sequences were a gift from Dr. L. McIntosh, Michigan State University. A plasmid containing *psbE/F* sequences was provided by Dr. H. Pakrasi, Washington University. Total cell lysates for immunoblot analysis were obtained by homogenization with glass beads (Burnap et al., 1989). Samples were solubilized and electrophoresed on denaturing 10–20% gradient polyacrylamide gels as described previously (Burnap et al., 1989) except that urea was omitted from the gel. Immunoblot analysis was performed using horseradish peroxidase conjugated goat anti-rabbit IgG. D1 and D2 antibodies (gifts from Dr. Y. Inoue, RIKEN Institute, Tokyo) and MSP antibodies (a gift from Dr. T. Kuwabara, Toho University) were directed against the homologous proteins isolated from spinach. Anti-CP47 antibody (a gift from Dr. N.-H. Chua, Rockefeller University) was directed against the homologue from *Chlamydomonas*.

Oxygen evolution was measured at 25 °C using a Clark-type electrode in a water-jacketed 2-mL stirred cuvette under

heat-filtered illumination transmitted through a red-pass filter (Corning 2406). The intensity of light impinging upon the the cuvette was approximately 2000 μEinsteins m^{-2} s^{-1}, which is above saturation of the O_2-evolving capacity of samples used in this work. Fresh cyanobacterial cultures in the mid-logarithmic growth phase were pelleted and resuspended to a chlorophyll concentration of 10 μg/mL in a reaction buffer consisting of 10 mM Hepes–NaOH, pH 7.2, and 10 mM NaCl. The electron acceptors were 15 mM $NaHCO_3$ or 0.6 mM 2,6-dichloro-*p*-benzoquinone and 5 mM $K_3Fe(CN)_6$. Inhibition of the H_2O-splitting enzyme in whole cells by hydroxylamine was performed as follows: cells from mid-logarithmic-phase cultures were resuspended to a concentration of 100 μg of Chl/mL in 10 mM HEPES–NaOH, pH 7.2, and 10 mM NaCl and incubated at room temperature in dim light for 10 min with varying concentrations of hydroxylamine (50–1200 μM). Samples were assayed for oxygen evolution immediately after the incubation period using the 2,6-dichloro-*p*-benzoquinone/$K_3Fe(CN)_6$ electron acceptor system as described above.

Fluorescence induction kinetic measurements were performed using a Walz pulse amplitude modulated (PAM) fluorometer (Schreiber et al., 1986) interfaced with an IBM PC. The measuring beam (PAM 101) was applied by using the auto-100-kHz function under conditions independently determined to be nonactinic (prior to triggering the actinic light which increased the sampling frequency), thus providing for an accurate measurement of F_0. Actinic light (λ_{max} = 650 nm) was provided by the PAM 102 LED at an intensity of approximately 25 μEinsteins m^{-2} s^{-1}. Due to the geometry of the instrument, concentrated samples (75 μg of chlorophyll/mL) were used.

Fluorescence emission spectra of whole cell samples were obtained at 77 K using an SLM 8000C spectrofluorometer fitted with a liquid N_2 optical dewar. Samples were prepared as follows: Mid-log-phase cells were harvested by low-speed centrifugation, resuspended in fresh media to a concentration of 30 μg of Chl/mL, and placed on a rotary shaker approximately 1.5 h under illumination at standard growth temperature. Samples were directly frozen as a thin coating on the outside of a liquid N_2 chilled NMR (2-mm) sample tube and used immediately for measurement.

Results

The *Synechocystis* sp. PCC6803 *psbO* gene was isolated as a 1.9-kb *Avr*II/*Hin*dIII fragment by probing a fragment-enriched plasmid library with the previously cloned *Synechococcus* PCC7942 *psbO* gene (Kuwabara et al., 1987) under conditions of reduced hybridization stringency. Sequence analysis of the isolated plasmid, designated pRB1, confirmed that the cloned fragment indeed contained the entire *psbO* gene as previously characterized by Philbrick and Zilinskas (1988). Furthermore, Southern blot analysis using conditions of reduced hybridization stringency confirmed previous results indicating that the *psbO* gene exists in single copy in the *Synechocystis* chromosome similar to the situation found in other species of cyanobacteria (Kuwabara et al., 1987; Borthakur & Haselkorn, 1989). To obtain a strain of *Synechocystis* sp. PCC6803 lacking the *psbO* gene, a mutagenic plasmid was constructed as described under Materials and Methods and shown in Figure 1A. After the wild-type cells were transformed with the mutagenic plasmid pRB42, several resultant transformant colonies were picked and subjected to repetitive restreaking on selective plates under conditions of photoheterotrophic growth. The transformants were also tested for the ability to grow in the presence of ampicillin (Ap) to reveal those which could have resulted from the integration of the entire plasmid by a single cross-over recombination. No Ap^r transformants were found, suggesting that the desired double cross-over/gene replacement event had occurred in each of the Sp^r isolates. Since *Synechocystis* sp. PCC6803 cells contain multiple copies of the genome, protracted growth on selective media is necessary for segregation of mutant alleles to obtain an isogenic strain (Williams, 1988). After 2 months of propagation in this manner, photosynthetically impaired

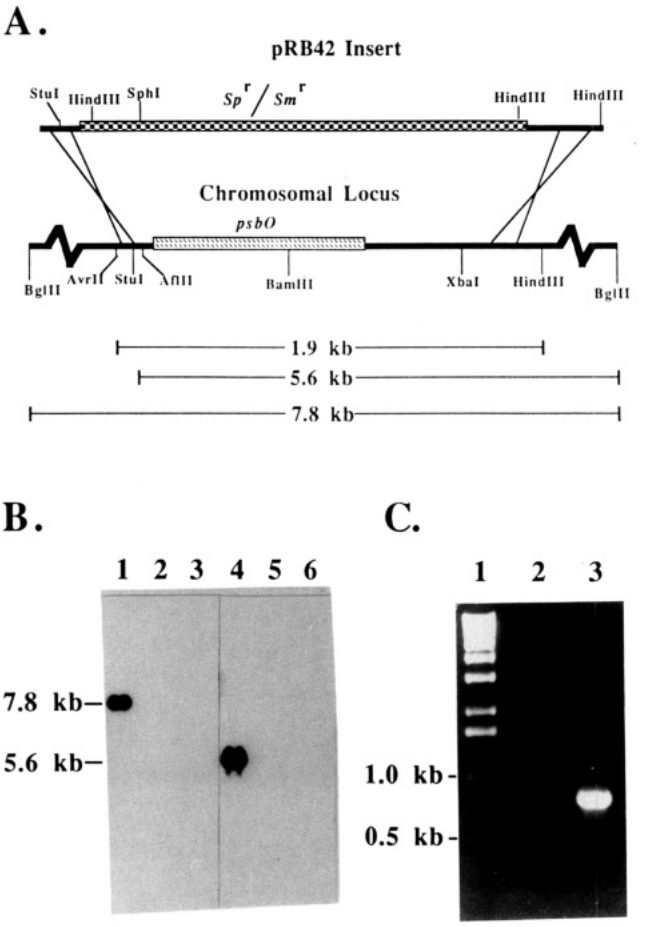

FIGURE 1: Construction of a *psbO* deletion mutant of *Synechocystis* sp. PCC6803. (Panel A) Strategy for the replacement of the *psbO* gene at its chromosomal locus with an antibiotic resistance cassette encoding spectinomycin (Sp) and streptomycin (Sm) resistances. The mutagenic plasmid pRB42 contains the Sp^r/Sm^r resistance cassette ligated between *Synechocystis* sequences which had previously flanked 5′ and 3′ ends of the cloned *psbO* gene. This plasmid, which is unable to replicate autonomously in *Synechocystis*, was used to transform the wild-type *Synechocystis* to Sp^r. The resultant transformants are expected to derive primarily from homologous integration events mediated via double cross-over recombination (Williams, 1988) at the *psbO* chromosomal locus as shown. (Panel B) Southern blot analysis of genomic DNA isolated from wild type (lanes 1 and 4) and Sp^r transformant S425 (lanes 2, 3, 5, and 6). Blot was probed with the [α-^{32}P]dCTP-labeled *Afl*II–*Xba*I fragment containing the *psbO* gene which had been deleted in the mutagenic plasmid pRB42. DNA has been digested with *Bgl*II (lanes 1–3) or *Bgl*III and *Stu*I (lanes 4–6). Lanes 3 and 6 contained DNA extracted from transformant S425 cells which had been extensively subcultured under autotrophic conditions without antibiotic selection. Southern analysis was performed by restriction digestion of 2-μg samples of genomic DNA followed by electrophoresis on a 0.8% agarose gel and blotting onto nitrocellulose. (Panel C) Polymerase chain reaction (PCR) analysis of genomic DNA isolated from the transformant S425 (lane 2) and wild type (lane 3) using oligonucleotide primers designed to amplify the 822 bp coding region of the *psbO* gene. Reaction products were electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining. Molecular weight markers ("1 kb ladder", BRL) are shown in lane 1.

mutants were selected by streaking the transformants on Sp-containing plates lacking DCMU and glucose, in addition to the routine restreaking on Sp/DCMU/glucose media. All the Sp^r transformants retained the ability to grow autotrophically, albeit more slowly, on solid media.

Since failure to find transformants incapable of autotrophic growth could, in principal, have several different explanations (e.g., incomplete segregation of the mutant allele), we undertook a characterization of the genotype by physical methods. Genomic DNA extracted from the wild type and one of the Sp^r isolates, designated S425, was subjected to Southern blot analysis using the *psbO*-containing *Afl*III/*Xba*I fragment (which had been depleted in pRB42) as a hybridization probe (Figure 1B). A 7.8-kb *Bgl*II fragment and a 5.4-kb *Bgl*II/*Stu*I fragment, which hybridize to the *psbO* probe in the wild-type samples, are absent in the transformant, indicating that the desired deletion of the *psbO* gene had occurred. The transformant was also allowed to grow autotrophically in liquid media without antibiotic selection for five consecutive subcultures (about 1 month total), which should confer a competitive growth advantage to cells still possessing the wild-type allele. Figure 1B shows that DNA extracted from the transformant grown under these conditions still lacks a detectable hybridizing band when probed with the cloned gene, thus arguing against the possibility that nonsegregant or contaminating wild-type cells were present in the isolate giving rise to autotrophic colonies.

A comparative analysis of the mutant and wild type using the highly sensitive polymerase chain reaction (PCR) technique further demonstrated that the *psbO* gene had been deleted in the mutant. Oligonucleotide primers initiating the synthesis of the *psbO* gene from opposite ends of the coding sequence were used in reactions involving 40 cycles of amplification beginning with 1 μg of genomic DNA extracted from either the wild type or the mutant S425 (grown autotrophically). These reaction conditions should allow the detection of trace amounts of the gene. As shown in Figure 1C, only the reaction containing wild-type DNA yielded the 822 bp *psbO* fragment, thus substantiating conclusions based upon Southern analysis that the *psbO* gene is absent in the Sp^r transformant strain.

The fact that the *psbO* deletion mutant grows autotrophically suggests that it is capable of assembling functional PSII in the absence of MSP. To investigate the expression of other PSII genes, as well as to confirm the absence of the expression of *psbO*, we performed Northern blot and immunoblot analyses of the mutant and wild type using the PSII gene and antibody probes available to us. Figure 2A shows that transcripts for the *psbA-2* gene (encoding D1) and the *psbC/D* operon (encoding CP43 and D2) are expressed in both the mutant and wild-type strains, whereas the 1.2-kb *psbO* transcript is absent in the mutant. The transcript for the *psbE/F* operon (encoding cyt b_{559}) is also expressed in the mutant (not shown). Interestingly, steady-state levels of the transcripts hybridizing with the *psbA-2* and *psbC/D* gene probes are higher in the mutant compared to the wild type. Similar results were obtained using the *psbE/F* probe (not shown). Whether or not this reflects an increased level of synthesis of the corresponding PSII proteins awaits further investigation. Immunoblot analysis of whole cell lysates of the mutant and wild type (Figure 2B) indicates that, although MSP is absent, wild-type levels of PSII proteins D1, D2, and CP47 accumulate in the mutant.

Measurements of the low-temperature fluorescence emission spectra of the mutant and wild type were performed to provide an indication of the state of PSII assembly. The 77 K fluorescence emission spectra of the mutant and wild type are

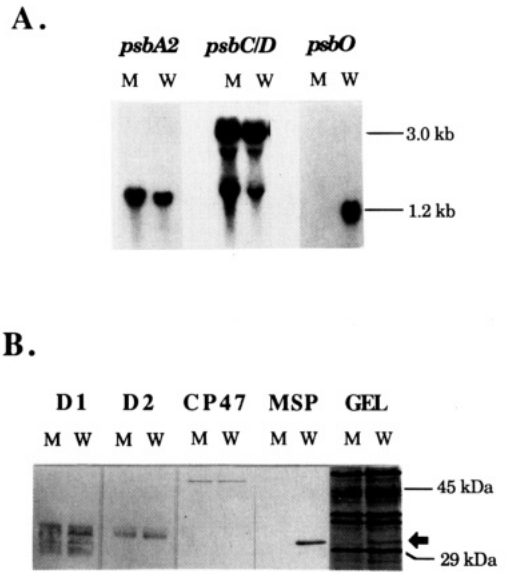

FIGURE 2: Northern and immunoblot analysis of photosystem II transcripts and proteins in mutant (M) and wild-type (W) cells. (Panel A) Total RNA probed with [α-^{32}P]dCTP-labeled restriction fragments containing cloned genes for the D1, D2 + CP43, and MSP proteins: *psbA-2*, *psbD1/C*, and *psbO*, respectively. Ten-microgram aliquots of cellular RNA were electrophoresed on a 1.2% agarose gel containing formaldehyde and blotted onto Nytran membranes for hybridization analysis. (Panel B) Immunoblotted total cell lysates reacted with antibodies directed against PSII proteins D1, D2, CP47, and MSP. The Coomassie-stained gel is shown on the right. Note the absence in mutant cells of a stained 32-kDa polypeptide which is presumed to be MSP (arrow).

qualitatively similar upon excitation of either chlorophyll (at 435 nm) (Figure 3A) or phycobilin (at 610 nm) (Figure 3B). This indicates that the accumulated PSII polypeptides assemble with the chlorophyll moieties of the complex [as judged by the characteristic emission at 686 and 695 nm (Sherman et al., 1987)] and that the complex remains energetically coupled with the light-harvesting phycobilisome. However, two notable differences between the mutant and wild type were consistently observed. First, in spectra obtained by using chlorophyll excitation (Figure 3A), the emission peak at 695 nm has a lower intensity in the mutant. Similarly, the emission peak at 692 nm and the shoulder at 695 nm, seen in the wild type upon phycobilin excitation, are both significantly diminished in the mutant. Second, in phycobilin-excited samples of the mutant, the yield of fluorescence arising from phycobilisome chromophores (650–670 nm) is greater than the wild type. It should be noted that the increased phycobilin fluorescence observed in the mutant is not simply due to elevated levels of phycobilin since the mutant and wild type have similar phycobilin contents and phycobilin:chlorophyll ratios under the mixotrophic (glucose + light) growth conditions used for these experiments (data not shown).

Although the *psbO* deletion mutant is capable of autotrophic growth, it does so at approximately 65% the rate of the wild type grown under similar conditions. The reduced growth rate of the mutant is paralleled by a rate of O_2 evolution which is 60–70% that found in the wild type when CO_2 is the terminal electron acceptor (Figure 4). A more pronounced difference in the rates of O_2 evolution between the mutant and wild type, approximately a factor of 3, is observed when the artificial electron acceptor system 2,6-dichloro-*p*-benzoquinone plus ferricyanide was used. Under these conditions, O_2 evolution for the wild-type is stimulated approximately 2.4-fold relative to the CO_2-supported rate, whereas this rate is only marginally stimulated in the mutant (Figure 4).

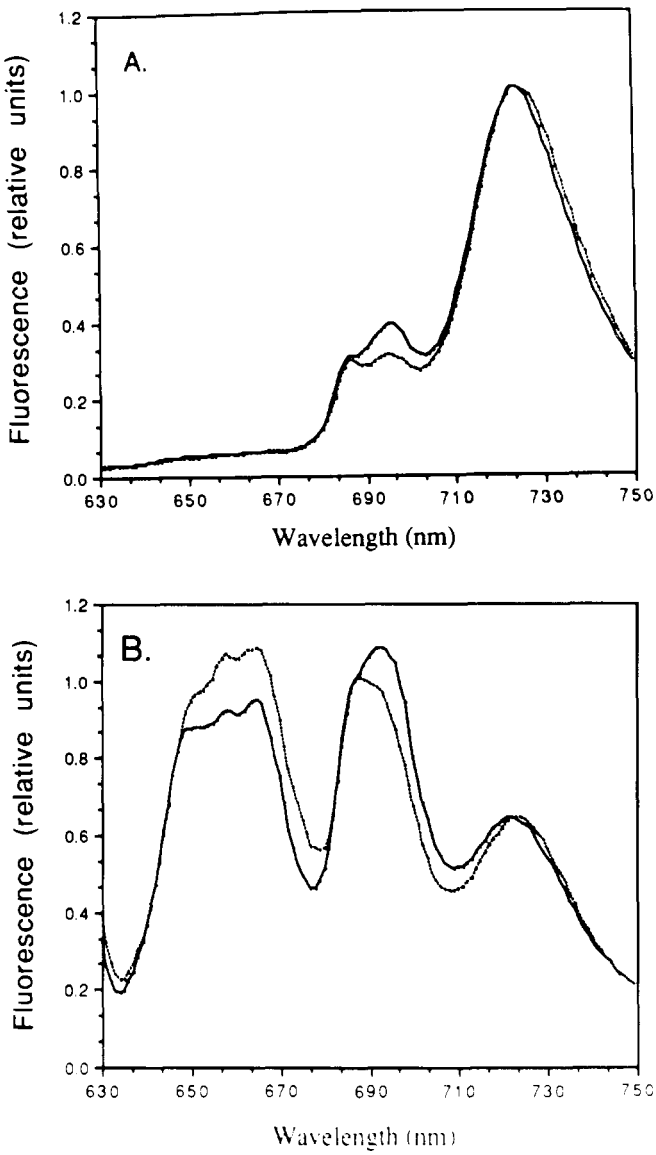

FIGURE 3: Corrected 77 K fluorescence emission spectra of wild-type (—) and mutant (···) cells. (Panel A) Spectra of samples excited at 435 nm (5-nm band-pass). (Panel B) Spectra of samples excited at 610 nm (5-nm band-pass).

Measurements of the increase in variable fluorescence yield in the presence of 20 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) were performed to better localize the lesion affecting PSII activity. These measurements (van Gorkom, 1986) assay the accumulation of the primary quinone electron acceptor, Q_A, in its reduced form, Q_A^-. A maximally fluorescent state (F_{max}) results from the accumulation of the [P680 Q_A^-] state where P680 is the primary electron donor of the reaction center. A lesion affecting the secondary electron donors (e.g., the Mn cluster) of PSII is expected to result in a reduced rate of electron donation to oxidized $P680^+$, thus allowing the charge recombination between $P680^+$ and Q_A^+ to effectively compete with the forward reaction. This would result in a low level of Q_A^- accumulation and a correspondingly low level of variable fluorescence. Indeed, the *psbO* deletion mutant exhibits a lower level of variable fluorescence suggestive of this situation (Figure 5). Furthermore, this fluorescence can be restored significantly by the addition of the reductant hydroquinone [which is capable of donating electrons to $P680^+$ (Debus et al., 1988; Metz et al., 1989)] (Figure 5), indicating that the reduced rate of H_2O-splitting activity observed polarographically is due to impaired enzyme

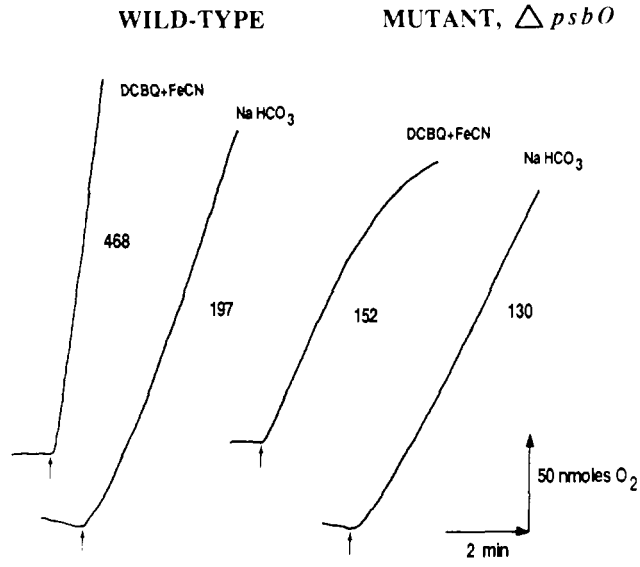

FIGURE 4: Oxygen evolution by wild-type and mutant cells using either CO_2 (as $NaHCO_3$) for the terminal electron acceptor or the artificial acceptor system 2,6-dichloro-*p*-benzoquinone plus ferricyanide (DCBQ+FeCN). Numerical values beside the representative traces indicate rates of O_2 evolution expressed as micromoles of O_2 per milligram of chlorophyll per hour. Arrows indicate the onset of illumination.

function on the electron donor side of PSII. For comparison, the fluorescence induction trace of a *Synechocystis* sp. PCC6803 strain which has the *psbE/F* operon deleted and exhibits no PSII activity (Pakrasi et al., 1989) is shown. As expected, the *psbE/F* deletion mutant exhibits no variable fluorescence even in the presence of hydroquinone (Figure 5B).

The H_2O-splitting activity of the mutant is also significantly more labile relative to the wild type as judged by the rapid and complete loss of activity during the application of procedures for the isolation of O_2-evolving membranes from cell lysates (Burnap et al., 1989). This increased lability presumably reflects a greater susceptibility of the Mn cluster to disruption in the absence of MSP. Evidence that the Mn cluster in the mutant is more accessible to the aqueous phase comes from experiments showing that submillimolar concentrations of hydroxylamine [previously shown to solubilize PSII-associated Mn (Ghanotakis et al., 1984; Tamura & Cheniae, 1985)] are more effective in inactivating O_2 evolution in the mutant as compared with the wild type. In these experiments (see Materials and Methods), cells were exposed to varying concentrations of hydroxylamine for 10 min and assayed for oxygen evolution in order to determine the concentration of hydroxylamine producing a 50% inhibition of the H_2O-splitting activity relative to the untreated rate (I_{50}). The I_{50} for the wild-type strain was 450 μM hydroxylamine, whereas the I_{50} for the *psbO* deletion strain was determined to be 130 μM. These results indicate that the mutant is approximately 3.5-fold more susceptible to hydroxylamine inhibition compared to the wild type and are consistent with the interpretation that the Mn cluster is more accessible to the aqueous phase in the absence of MSP. Efforts to stabilize the H_2O-splitting activity of the mutant during membrane isolation (e.g., including high Cl^- concentrations in the isolation buffers) have thus far been unsuccessful.

Discussion

In this paper, we describe the construction and analysis of a strain of *Synechocystis* sp. PCC6803 in which the entire *psbO* gene encoding MSP has been deleted. Despite the complete absence of MSP, the mutant strain is capable of

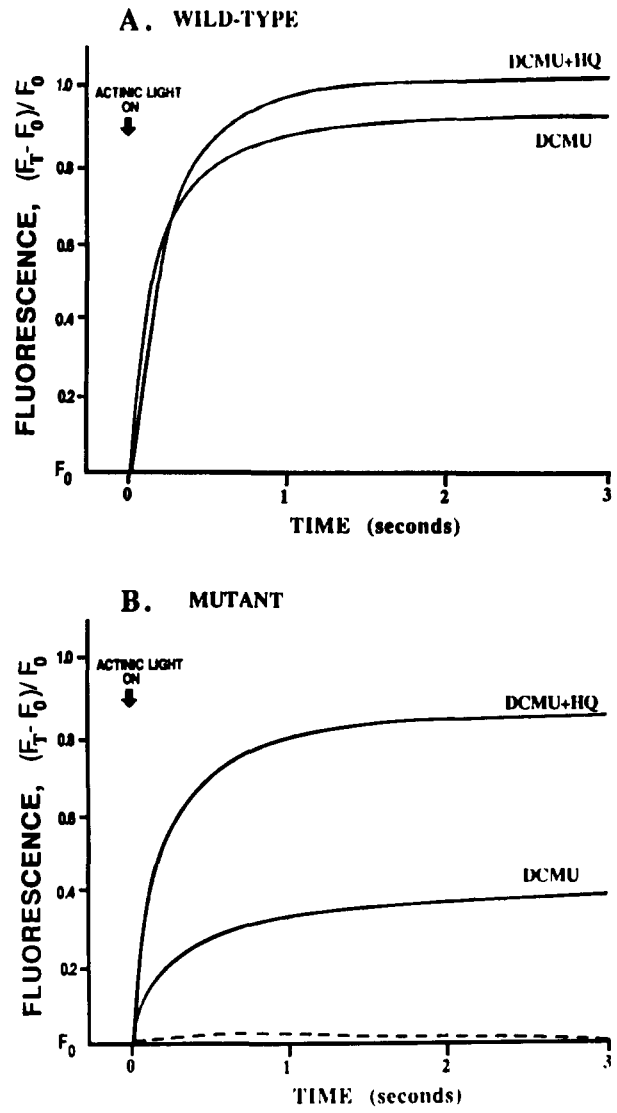

FIGURE 5: Increase in the fluorescence yield of chlorophyll in wild-type (panel A) and *psbO* deletion mutant (panel B) cells in the presence of 20 μM DCMU. Hydroquinone (HQ) was added to a concentration of 2.5 mM where indicated. For reference, the dashed line in panel B shows the fluorescence induction profile (in the presence of DCMU and HQ) of a *Synechocystis* sp. PCC6803 strain in which the *psbE/F* operon is deleted. This strain exhibits no PSII activity (Pakrasi et al., 1989). Fluorescence is plotted as $(F_{total} - F_0)/F_0$. Under the conditions used, the fluorescence yield reached greater than 90% F_{max} for both the mutant and wild type.

autotrophic growth, assembles functional PSII complexes, and retains the ability to evolve O_2, albeit at reduced rates. The finding that the *psbO* deletion mutant assembles active PSII complexes stands in sharp contrast to *Synechocystis* sp. PCC6803 PSII mutant strains constructed by the interruption or deletion of PSII genes encoding membrane-spanning proteins. In these cases, the effects of gene inactivation are generally pleiotrophic with phenotypes exhibiting a reduction or complete loss in the accumulation of other PSII gene products, impaired formation of the PSII complex, and a concomitant loss in the ability to catalyze the H_2O-splitting reaction (Vermass et al., 1986, 1988; Pakrasi et al., 1989).

From our results, it is obvious that MSP is not essential for significant rates of H_2O-splitting activity to occur in vivo, at least in this species of cyanobacterium. The *psbO* deletion mutant evolves O_2 at approximately one-third the rate of the wild type under conditions employing an artificial acceptor system intercepting electrons at the acceptor side of PSII (see Figure 4). The fact that H_2O-splitting activity is possible in the complete absence of MSP is particularly significant relative to previous biochemical investigations of MSP. Treatment of PSII preparations in vitro with 1 M $CaCl_2$ results in the liberation of MSP yet preserves active-site Mn (Ono & Inoue, 1984). The $CaCl_2$-treated preparations exhibit low rates of O_2 evolution (about 20% of the original, untreated preparation) when provided with high Cl^- concentrations, and a significant level of the original activity is restored by the rebinding of MSP (Kuwabara et al., 1985). While the existence of measurable rates of O_2 evolution in $CaCl_2$-treated PSII preparations suggests that MSP is not essential for catalytic activity, these preparations were shown to contain residual amounts of MSP, and it could be argued that the observed activity is due to PSII centers retaining MSP (Camm et al., 1987). This latter possibility is rendered more plausible given the uniformity of previous results (discussed below) indicating that MSP is required for O_2 evolution in vivo (Mayfield et al., 1987; Philbrick & Zilinskas, 1988; Mizobuchi & Yamamoto, 1989). In contrast, the results presented here clearly provide independent support of the former suggestion that MSP is not essential for O_2 evolution but does promote optimum activity.

Two alternative, but not mutually exclusive, hypotheses, can be invoked to account for the lower rates of O_2 evolution by the mutant. First, there are fewer PSII reaction centers capable of H_2O splitting present in the thylakoid membrane of the mutant. Since the MSP-less mutant accumulates wild-type levels of the intrinsic PSII polypeptides (identified immunologically, Figure 2B), this would imply that a proportion of these accumulated proteins are not associated with O_2-evolving complexes. A second hypothesis is that the rate at which the H_2O-splitting enzyme turns over is retarded for individual PSII reaction centers due to the absence of MSP. This possibility is consistent with the report that MSP accelerates the S-state transition from S_3–S_0 of the catalytic cycle (Miyao et al., 1987). Experiments designed to gain an understanding of this question, as well as the nature of the alteration in the donor side electron transport chararacteristics evidenced by the fluorescence induction experiments, are currently in progress.

While the *psbO* deletion mutant assembles active PSII complexes, fluorescence emission spectra at 77 K (Figure 3) indicate that the absence of MSP results in significant changes in the organization of chromophores of the PSII complex and its associated antennae. Most notably, we observe a reduction in the 695-nm emission signal which can be attributed to an alteration in the fluorescence of proximal PSII antennae chlorophyll associated with CP47 (Sherman et al., 1987). The reduction of this emission suggests that the binding of MSP to the lumenal portion of the PSII complex may alter the conformation of CP47 or its interaction with other components in the membrane (e.g., the D1/D2 heterodimer). Nearest-neighbor analyses using monoclonal antibodies (Bricker & Frankel, 1987) and bifunctional cross-linking reagents (Enami et al., 1990) have indicated that MSP is in contact with CP47, which is consistent with the interpretation that the reduction in the 695-nm emission is due to the loss of an intermolecular contact between MSP and CP47 which normally stabilizes the conformation (and consequently the fluorescence properties) of the latter. Alternatively, the level of CP47 assembled with chlorophyll may be reduced in mutant cells. While this possibility has not yet been rigorously excluded, it appears less likely given the fact that wild-type levels of the CP47 apoprotein accumulate in mutant cells as judged by immunoblot analysis (Figure 2B).

The fact that *Synechocystis* sp. PCC6803 is capable of autotrophic growth and O_2 evolution in vivo in the absence

of MSP is intriguing in view of previous investigations indicating the contrary. In eukaryotic algae, MSP has been shown to be required for the stable assembly of the PSII complex (Mayfield et al., 1987; de Vitry, 1989) and for the capacity to evolve O_2 in vivo (Mayfield et al., 1987; Mizobuchi & Yamamoto, 1989). This points to the existence of significant interspecies differences with respect to the function and assembly of the PSII reaction center and/or the subcellular context in which it operates. A conspicuous difference between cyanobacteria and eukaryotes is that the latter possess two additional extrinsic polypeptides (16 and 23 kDa) which are associated with the H_2O-splitting region of PSII and which enhance the binding of the inorganic cofactors Ca^{2+} and Cl^- necessary for H_2O-splitting activity (Babcock et al., 1989). Conversely, a 9-kDa extrinsic polypeptide associated with the lumenal portion of PSII in cyanobacteria has been found to be critical for O_2 evolution, but has no recognized counterpart in eukaryotes (Wallace et al., 1989). The extent to which these structural differences on the oxidizing side of PSII relate to the observed differences in stability and activity of the PSII complex remains to be established. Clearly, the possibility that immunologically distinct proteins are capable of replacing MSP in cyanobacteria warrants further investigation.

We also note that the insertional inactivation of the *psbO* gene in *Synechocystis* sp. PCC6803 was reported to result in a strain incapable of autotrophic growth (Philbrick & Zilinskas, 1988). However, that phenotype evidently is due to a secondary mutation affecting PSII function, and more recent results from that laboratory are consistent with those presented here (Dr. B. Zilinskas, personal communication). In conclusion, our results concur with biochemical depletion/reconstitution studies (Ono & Inoue, 1984; Kuwabara et al., 1985) which have suggested that MSP promotes optimal H_2O-splitting activity by PSII but is not absolutely required for significant rates of catalysis to occur. Given these results, it is tempting to speculate that the integral membrane polypeptides of the reaction center provide an environment sufficient for binding of the Mn cluster and for the catalysis of H_2O splitting, although the 33-kDa MSP protein may be necessary for optimum activity and stability.

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Registry No. O_2, 7782-44-7.

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