Oxygen Yield and Thermoluminescence Characteristics of a Cyanobacterium Lacking the Manganese-Stabilizing Protein of Photosystem II†

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ABSTRACT: Previous experiments have shown that a *Synechocystis* sp. PCC 6803 mutant (Δ*psbO*) lacking the extrinsic manganese-stabilizing protein (MSP) exhibits impaired, but significant levels of H_2O-splitting activity [Burnap, R., & Sherman, L. A. (1991) *Biochemistry 30*, 440–446]. [^{14}C]DCMU-binding experiments now show that the number and affinity of DCMU-binding sites (normalized to chlorophyll) are equivalent in Δ*psbO* and the wild type, suggesting equal concentrations of assembled reaction centers. A similar conclusion is reached on the basis of measurements of PSII electron transport (DPC-supported DCPIP reduction) by mutant and wild-type thylakoids. The pattern of flash O_2 yield by Δ*psbO* cells measured with a bare platinum electrode exhibits a period four oscillation (with a maximum on the third flash), indicating that the H_2O-splitting enzyme in Δ*psbO* retains the basic mechanistic features found in normal cells. However, the amplitude of these signals is smaller and more highly damped than those obtained from wild-type cells, suggesting the absence of MSP results in a higher miss probability and/or a reduction in the number of centers competent in oxygen evolution. Analysis of the rise kinetics of the ampermeric signal on the bare platinum electrode indicates that the S_3–[S_4]–S_0 transition is retarded by at least a factor of 5 in the mutant. Thermoluminescence emission peak temperatures indicate that the $S_2Q_A^-$, $S_2Q_B^-$, and $S_3Q_B^-$ charge pairs are significantly more stable with respect to recombination in the mutant. The intensities of the thermoluminescence emissions are also significantly reduced in the mutant. Taken together, the data suggest that functional consequences of the genetic removal of MSP are complex. Although the number of photochemically active PSII reaction centers is not much changed by the absence of MSP, the proportion of centers which are coupled to functional O_2-evolving enzymes appears to be reduced. For those centers which are effectively coupled to O_2 evolution, we find evidence of alterations in the kinetic properties of the enzyme due to the absence of MSP. These are (1) an increased miss factor, (2) a retardation of the S_3–[S_4]–S_0 transition, and (3) an increase in the stabilization of the S_2 and S_3 states.

The H_2O-splitting reaction of oxygenic photosynthetic is catalyzed by the membrane-bound photosystem II (PSII)[1] complex. The oxidative decomposition of two molecules of H_2O involves the utilization of four oxidizing equivalents accumulated in the H_2O-splitting enzyme as a result of four successive charge-separation and -transfer events within the PSII reaction center. Accordingly, the H_2O-splitting enzyme passes through a series of oxidation states, termed S states, which correspond, at least in part, to the stepwise oxidation of a cluster of four Mn atoms believed to form the catalytic center of the H_2O-splitting reaction [For recent reviews on PSII, see Babcock et al. (1989), Bruvig, et al. (1989), Ghanotakis and Yocum (1990), and Hansson and Wydrzynski (1990)].

The structure of the protein environment forming the active site of the H_2O-splitting enzyme remains to be elucidated. Current evidence suggests that the atoms of the Mn cluster are ligated by one or more of the intrinsic polypeptides of the PSII complex in a region accessible to the aqueous space of the thylakoid lumen (Andersson et al., 1987; Seibert et al., 1989; Mei et al., 1989; Svensson et al. 1990). In addition to the intrinsic PSII polypeptides, one or more extrinsic polypeptides are associated with the H_2O-splitting portion of the PSII complex [see Ghanotakis and Yocum (1990)]. Three such polypeptides with approximate molecular weights of 33 000, 24 000, and 18 000 have been identified in higher plants and eukaryotic algae. Cyanobacteria appear to lack homologs for the 24- and 18-kDa extrinsic polypeptides, while a homolog for the 33-kDa extrinsic polypeptide has been found in all species examined to date.

The 33-kDa extrinsic polypeptide has been referred to as the Mn-stabilizing protein (MSP) on the basis of biochemical depletion/reconstitution experiments showing that it stabilizes the binding of two of the four active site Mn atoms (Ono & Inoue, 1984). The loss of active site Mn, following biochemical removal of MSP, can be suppressed by the presence of high concentrations (>100 mM) of Cl^- (Kuwabara et al., 1985).

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[1] Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; MES, 2-(*N*-morpholino)ethanesulfonic acid; MSP, manganese-stabilizing protein, extrinsic 33-kDa PSII protein; Q_A, primary plastoquinone electron acceptor; Q_B, secondary, exchangeable plastoquinone electron acceptor; P680, photooxidizable chlorophyll species acting as primary electron donor of the reaction center; PSII, photosystem II; *psbO*, gene encoding the manganese-stabilizing protein; S_n, oxidation states of the H_2O-splitting enzyme, where *n* represents the number of stored oxidizing equivalents; Y_Z, redox-active tyrosine of the D1 protein acting as secondary electron donor of the reaction center.

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High concentrations of Cl^- can also restore a significant fraction of the H_2O-splitting activity that is otherwise nearly absent in PSII preparations depleted of MSP, which indicates that MSP is not absolutely essential for the catalytic activity of the H_2O-splitting enzyme (Kuwabara et al., 1985). Examination of the catalytic properties of MSP-depleted and reconstituted PSII preparations indicates that the removal of MSP leads to enhanced stability of the S_2 state (Miyao et al., 1987; Vass et al., 1987) and the inhibition (Ono & Inoue, 1985) or retardation (Miyao et al., 1987) of the S_3–$[S_4]$–S_0 transition. Whether or not the S_3–$[S_4]$–S_0 transition is completely blocked or simply retarded appears to depend upon the absence or presence, respectively, of high concentrations of Cl^- ions (Miyao et al., 1987).

A strain of the cyanobacterium *Synechocystis* sp. PCC6803 was constructed with the entire *psbO* gene, which encodes MSP, eliminated from its chromosomal locus by means of directed deletion mutagenesis (Burnap & Sherman, 1991). The resultant mutant, Δ*psbO*, was found to retain the ability to evolve O_2, albeit at reduced rates, despite the total absence of MSP. Similar results have been obtained independently, thus reinforcing the conclusion that MSP is not absolutely essential for O_2 evolution in this species of *Synechocystis* (Philbrick et al., 1991; Mayes et al., 1991). In a more recent development, it was shown that the *psbO* gene can also be inactivated in the nonphotoheterotrophic cyanobacterium *Synechococcus* sp. PCC7942 without the total loss of O_2-evolving activity (Bockholt et al., 1991). While these findings are consistent with the aforementioned biochemical results, it should be noted that they contrast with results indicating that MSP is required for O_2 evolution by eukaryotic algae in vivo. Specifically, it has been found that a *Chlamydomonas* mutant deficient in MSP does not evolve oxygen (Mayfield et al., 1987), and in *Euglena*, a correlation between the acquisition of oxygen evolution activity and the synthesis of MSP is reported (Mizobuchi & Yamamoto, 1989). The basis for these contrasting results remains to be clarified.

The rate of oxygen evolution by the *Synechocystis* strain Δ*psbO* under continuous saturating light is about $^2/_3$ the rate of the wild type under conditions where electron transport from substrate H_2O is coupled to carbon fixation (Burnap & Sherman, 1991; Philbrick et al., 1991; Mayes et al., 1991). The difference between the mutant and wild type is much more pronounced when DCBQ is employed as an electron acceptor (intercepting electrons from the Q_B site of PSII). Under these conditions, the rate of O_2 evolution by the wild type is stimulated approximately 2.5-fold relative to the coupled rate, while the rate of the mutant is only slightly enhanced (Burnap & Sherman, 1991). Fluorescence yield measurements performed in the presence of DCMU show that the mutant exhibits a lower fluorescence yield which can be restored to levels approaching those of the wild type by addition of the artificial electron donor hydroxylamine (Burnap & Sherman, 1991; Philbrick et al., 1991). These results suggest that the reduced rate of O_2 evolution is due an alteration in the donor side properties of the mutant enzyme. Inactivation of the *psbO* gene has also been shown to result in a marked increase in susceptibility to photoinactivation (Philbrick et al., 1991; Mayes et al., 1991) as well as an enhanced demand for Ca^{2+} ion for cell growth in culture (Philbrick et al., 1991).

In the present study, we have further investigated PSII in Δ*psbO* in terms of the following: (1) quantifying the concentration of photochemically active PSII reaction centers in the mutant relative to the wild type and (2) the O_2 evolution characteristics of the mutant enzyme using flash oxygen yield and thermoluminescence techniques. Our observations are consistent with the interpretation that the loss of MSP results in a retardation of one or more of the S-state transitions and a reduced efficiency in the utilization of oxidizing equivalents generated at the reaction center (higher miss probability) as the mutant enzyme is advanced through the S states using brief saturating flashes. Furthermore, compared to the wild type, the S_2 and S_3 states of the mutant were found to be significantly more stable with respect to recombination with negative charges present on the acceptor side of the PSII reaction center.

MATERIALS AND METHODS

Growth and maintenance of the glucose-utilizing strain of *Synechocystis* sp. PCC6803 (subsequently referred to as the wild type) and the derivative strain Δ*psbO* were conducted using established procedures (Williams, 1988). Construction of, and initial characterization of, Δ*psbO* has been described previously (Burnap & Sherman, 1991). For the measurements described here, the wild-type and mutant strains were grown in batch culture in the presence of 5 mM glucose. Cells were harvested during the late logarithmic phase of growth as opposed to being harvested during midlogarithmic growth as described previously (Burnap & Sherman, 1991). This modification was found to improve the stability of the photosynthetic activity of the mutant strain, which is particularly prone to decay.

Flash oxygen yield was measured using a bare platinum Joliot-type electrode with an AC-coupled transimpedence amplifier with a 0.5-ms rise time in an apparatus described previously (Jursinic & Dennenberg, 1990). The platinum surface of the electrode was scrupulously cleaned with a $CaCO_3$ paste between measurements. Cell suspensions at a concentration of 175 μg of Chl/mL were introduced into the sample channel and allowed to dark adapt for 10 min. The platinum electrode was polarized to –0.7 V relative to the Ag/AgCl electrode immediately prior to the flash sequence. Oxygen evolution by whole cells under continuous illumination and using DCBQ/$K_3Fe(CN)_6$ as the artificial acceptor system was measured with a Clark-type concentration electrode as described previously (Burnap & Sherman, 1991).

Measurements of DPC-supported DCPIP reduction were made on isolated thylakoids. Thylakoids were isolated from cells using a modification of a method described previously (Burnap et al., 1989). Briefly, cells harvested from 250 mL of late logarithmic phase cultures were resuspended in approximately 5 mL of MMCS buffer (50 mM MES/NaOH, pH 6.5, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 0.8 M sucrose) containing 500 mM NaCl (MMCS/HS) and placed on ice in the dark for approximately 1 h. The suspended cells were repelleted and resuspended in the same buffer to a concentration of 800 μg of Chl/mL. A 1-mL aliquot of the resuspended cells was transferred to a 2-mL screw-capped microfuge tube, and glass beads (0.1 mm) were added to the tube such that a small airspace remained once the tube was closed. The tube was then secured on a modified vortex shaker, and the contents were agitated for 5 min at top speed under a cooling stream of CO_2 gas from a siphon tank. Glass beads, unbroken cells, and debris were pelleted from the cell homogenate by centrifugation at low speed in a microfuge. The glass beads were extracted two to three times with fresh buffer and pelleted as before to recover trapped thylakoids. When combined with the first supernatant, the total volume of the combined supernatants was 3 mL. Membranes were pelleted from the combined supernatants by centrifugation at 150000*g* in a

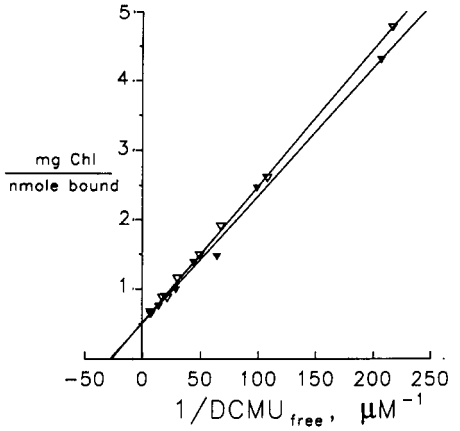

FIGURE 1: Double-reciprocal plot of [^{14}C]DCMU binding to wild-type (open triangles) and Δ*psbO* (closed triangles) cells.

benchtop ultracentrifuge for 30 min. At this stage, membranes isolated from both wild-type and Δ*psbO* cells retain a significant fraction (>70%) of the DCBQ-supported O_2 evolution activity present in the cells used as starting material. However, the activity of membranes isolated from Δ*psbO* cells is much more labile than in preparations from the wild type. Refinement of this method and full characterization of O_2-evolving activity in the resultant preparations is currently in progress. Immediately prior to DPC-supported DCPIP reduction assays, the membranes were resuspended to a concentration of 400 mg of Chl/mL and treated with 2 mM hydroxylamine for 30 min on ice to extract active site manganese. After the membranes were pelleted and resuspended in fresh MMCS, the rates of DPC-supported DCPIP reduction were assayed spectroscopically using a Beckman DW-2 spectrophotometer essentially as described by Vermaas et al. (1990).

Thermoluminescence measurements of whole cells were performed essentially as described previously except that samples were resuspended in a buffer consisting of 10 mM NaCl and 10 mM MES/NaOH, pH 6.5, and dark adapted on ice for 15 min (instead of at room temperature). This dark-adaptation protocol was found to provide the most uniform results when used with cells grown in the presence of glucose. Samples were excited with a saturating xenon flash and cooled rapidly in liquid N_2, and the light emission during sample warming (approximately 1 °C/s) was recorded against sample temperature as described previously) Govindjee et al., 1985).

Estimation of the number of herbicide-binding sites (on a per chlorophyll basis) and the DCMU binding affinity of these sites in whole cyanobacterial cells was performed using the [^{14}C]DCMU-binding assay described previously (Vermaas et al., 1990). [^{14}C]DCMU was added as an ethanolic solution, and all samples were adjusted to equivalent concentrations of ethanol (5% v/v). Chlorophyll concentration was determined using the equations of McKinney (McKinney, 1941).

RESULTS

The results of [^{14}C]DCMU-binding assays performed upon Δ*psbO* and wild-type *Synechocystis* sp. PCC6803 cells and expressed in the form of double-reciprocal plots are shown in Figure 1. No significant differences between the mutant and the wild type are observed by this method with respect to either the number of herbicide-binding sites (on a per chlorophyll basis) or the DCMU binding affinity of these sites (Table I). This experiment suggests (1) that the loss of

Table I: Measurements DCMU Binding Sites[a] and PSII Electron Transport[b,c] in Δ*psbO* and Wild-Type Samples

	Chl/DCMU site[a]	K_D DCMU[a] (nM)	DPC → DCPIP[b]	H_2O → DCBQ[c]
wild type	565	39	148	295
Δ*psbO*	550	36	156	106

[a] The number (Chl/site) and affinity (K_D) of DCMU-binding sites in mutant and wild-type cells were estimated from the *Y* intercepts and *X* intercepts of the double-reciprocal plots shown in Figure 1. [b] Electron transport was measured in hydroxylamine-extracted thylakoids (see Materials and Methods) from the mutant and wild type by monitoring DCPIP reduction spectrophotometrically at 590 nm. Rates are expressed as μmol of DCPIP reduced·mg of $Chl^{-1}\cdot h^{-1}$. The reaction medium consisted of HMCS buffer containing 100 μM DCPIP, 500 μM DPC, and thylakoids at a concentration of 5 μg of chlorophyll/mL. [c] Oxygen evolution (μmol of O_2·mg of $Chl^{-1}\cdot h^{-1}$) under continuous saturating illumination was measured at 25 °C with the addition of 600 μM DCBQ, 5 mM $K_3Fe(CN)_6$, and cyanobacterial cells at a concentration of 10 μg of chlorophyll/mL.

the *psbO* gene and the consequent absence of MSP in mutant cells does not appreciably affect the steady-state level of assembled PSII reaction centers relative to the wild type and (2) that the loss of MSP binding to the reaction center does not result in significant conformational alterations of the DCMU-binding region of the PSII complex.

Measurements of light-driven transport of electrons from DPC to DCPIP were performed to provide an estimate of the relative concentrations of photochemically active PSII complexes in thylakoids isolated from mutant and wild-type cells. The results of these measurements, summarized in Table I, show that the thylakoids of the mutant exhibit approximately the same level of DPC-supported DCPIP reduction activity as those from the wild type, indicating that the mutant and wild type contain equivalent concentrations of photochemically active PSII reaction centers on a per chlorophyll basis. In contrast, the mutant exhibits a much lower rate of oxygen evolution compared to the wild type under continuous saturating illumination using DCBQ and potassium ferricyanide as the acceptor system (Table I). A similar disparity in relative rates between the mutant and wild type was observed previously (Burnap & Sherman, 1991), although the absolute values are different due to differences in the point in the growth phase when cells were harvested (Kawamura et al., 1979).

The O_2 yield patterns of cells applied to a bare platinum Joliot-type electrode (Joliot & Joliot, 1968; Jursinic & Dennenberg, 1990) and illuminated with a sequence of short saturating flashes applied at 2 Hz are shown in Figure 2. Oxygen yield by the mutant oscillates as a function of flash number with a periodicity of four, indicating that the H_2O-splitting reaction catalyzed by mutant reaction centers resembles the normal process mechanistically. However, the magnitude of the oxygen signals from the mutant during the flash sequence is consistently smaller than those observed for the wild type measured under similar conditions. After 25 flashes, where all oscillation in yield is effectively damped out due to mixing of S states, the amplitude of the mutant signal is constant (not shown) but only 20–40% the amplitude of the signal obtained from wild-type cells. It is important to note that the large variability in relative amplitudes between the mutant and wild type (20–40%) reflects a variability in the properties of samples of mutant cells. In contrast, the amplitude of the oxygen yield signals obtained from wild-type samples was highly reproducible during the repetition of these experiments, provided that we rigorously adhered to the conditions described in Materials and Methods. The source of the variability observed for samples of the mutant is under

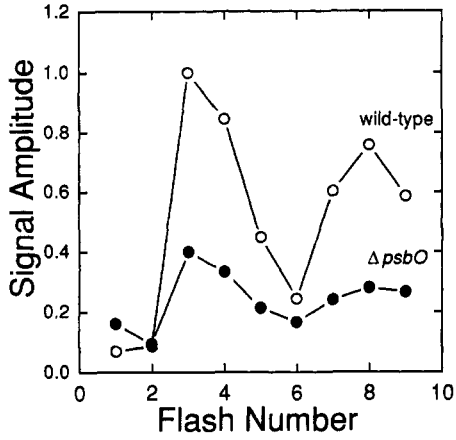

FIGURE 2: Oxygen production by dark-adapted wild-type (open circles) and Δ*psbO* (closed circles) cells as a function of flash number. Cells were applied to a bare platinum electrode, dark adapted for 10 min, and given a train of saturating light flashes at 2-Hz frequency.

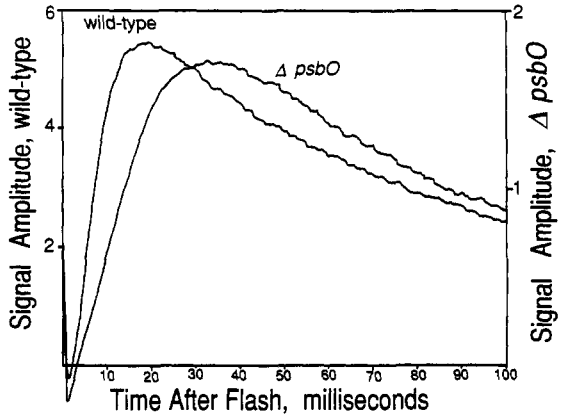

FIGURE 3: Oxygen signals of wild-type and Δ*psbO* cells on a bare platinum electrode illumiated with saturating xenon flashes. The signals shown are for cells after 25 flashes have been given. The exponential rise times of the depicted wild-type and Δ*psbO* signals are 5.3 and 16.9 ms, respectively. The corresponding exponential decay times for the wild-type and Δ*psbO* signals are 104 and 115 ms, respectively. Kinetic analysis of the data was performed according to the exponential method described in Jursinic and Dennenberg (1990).

investigation, but it is apparently not due exclusively to an enhanced susceptibility to photoinactivation since similar trends in variability were seen with a range of light intensities under which samples were grown and prepared.

Part of the reduction in apparent flash oxygen yield of Δ*psbO* observed using the bare platinum electrode is likely due to a higher miss probability as evidenced by the higher degree of damping of the oscillation of the oxygen signal. Matrix analysis of the oscillation patterns (Lavorel, 1976) gives estimates for the average miss probabilities (α) for successive S-state transitions for the wild type and mutant as 0.15 and 0.22, respectively. The larger average miss probability estimated for the mutant suggests that positive charges generated in the mutant reaction centers are less efficiently utilized for the advancement of the H_2O splitting through the sequence of S states leading to oxygen evolution.

Kinetically resolved oxygen signals typical of mutant and wild-type samples measured using the bare platinum electrode are shown in Figure 3. The rise of the mutant signal is consistently found to be slower than that seen for the wild type measured under identical conditions. Analysis of oxygen signals, assuming single exponentials for their rise and decay, has been found to provide a reasonable fit to experimental

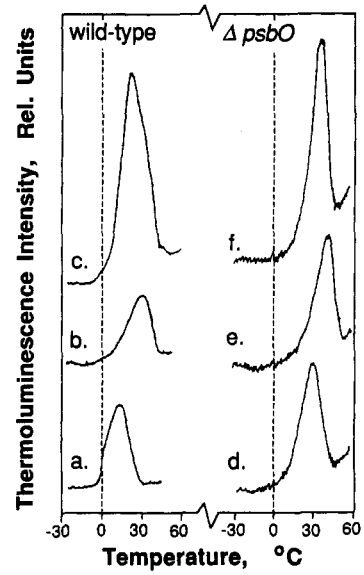

FIGURE 4: Thermoluminescence emission elicited by flash illumination of wild-type (traces a–c) and Δ*psbO* (traces d–f) cells. Traces a and d are of wild-type and Δ*psbO* samples, respectively, treated with 20 μM DCMU and given one xenon flash prior rapid immersion in liquid N_2 and followed by measurement of light emission during rewarming of the samples (1 °C/s). Traces a and d correspond primarily to the recombination of the $S_2Q_A^-$ charge pair. Traces b and e (wild type and Δ*psbO*, respectively) are of samples given one flash in the absence of DCMU and correspond primarily to recombination of the $S_2Q_B^-$ charge pair. Traces c and f (wild type and Δ*psbO*, respectively) are of samples given two flashes in the absence of DCMU and correspond primarily to the recombination of the $S_3Q_B^-$ charge pair. The glow curves of the mutant samples were recorded at 5× greater sensitivity than those of the wild-type samples.

data obtained using the bare platinum electrode employed here (Jursinic & Dennenburg, 1990). Applying this analysis, the mean exponential rise times for the mutant and wild-type signals were found to be 5.4 and 14.3 ms, respectively, whereas the decays for the two samples were found to be experimentally indistinguishable and in the range of 100 ms. We postulate that rise of the oxygen signal depends upon the rate constant of oxygen release by the H_2O-splitting enzyme and on the diffusion time of oxygen out of the cell and through the intervening buffer to the electrode surface. Since mutant and wild-type samples were treated identically and there is presently no reason to expect that the parameters governing diffusion should be different between the two types of sample measured here, we interpret the slower rise of the oxygen signal from flashed Δ*psbO* cells to indicate that the S_3 to S_0 transition is slower in the mutant than in the wild type.

Thermoluminescence (TL) by photosynthetic material is light emitted during the rewarming of samples which have been first exposed to actinic illumination and then frozen [reviewed in Sane and Rutherford (1986) and Vass and Inoue (1992)]. For the experiments described here, TL arises almost exclusively from PSII and is due to the thermally stimulated recombination of pairs of opposite charges (e.g., $S_2Q_A^-$) generated during light-induced charge separation by the PSII complex. Thermoluminescence emission following single flash excitation of dark-adapted cyanobacterial cells results largely from the recombination of the $S_2Q_B^-$ charge pair ("B band") (Govindjee et al., 1985). If electron transfer between Q_A and Q_B is blocked prior to excitation by an inhibitor such as DCMU, then thermoluminescence arises from the recombination of the $S_2Q_A^-$ charge pair ("Q band"). Figure 4 shows the TL glow curves of Δ*psbO* and the wild type. Wild-type cells exhibit TL emission maxima for the $S_2Q_A^-$ and $S_2Q_B^-$ charge

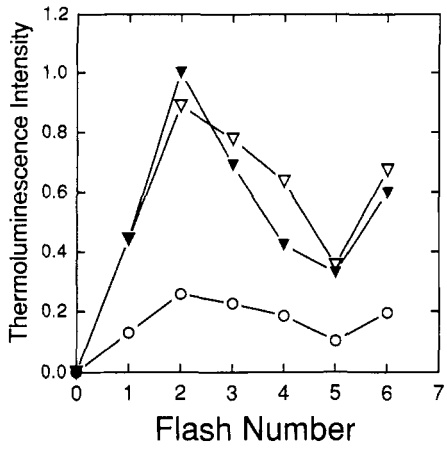

FIGURE 5: Oscillation of the flash-induced thermoluminescence B band from wild-type (open symbols) and Δ*psbO* samples (closed symbols): B-band height from wild-type cells as a function of the number of flashes given prior to measurement (closed triangles); B-band height from Δ*psbO* cells as a function of the number of flashes given prior to measurement (open circles); normalized B-band height from Δ*psbO* cells (open triangles) with the B-band height on the first flash normalized with that of the wild type.

recombinations (traces a and b, respectively) at approximately 12 and 31 °C, respectively. In contrast, the corresponding peak emission temperatures of the *psbO* deletion mutant are upshifted, relative to the wild type, to 26 and 39 °C for the $S_2Q_A^-$ and $S_2Q_B^-$ charge recombinations (traces d and e, respectively). Since the temperature at which maximal luminescence occurs is a function of the free energy of stabilization of the charge-separated state, the relative upshifts in the peak temperatures of the $S_2Q_A^-$ and $S_2Q_B^-$ recombinations observed for the deletion mutant indicate that the loss of MSP increases the stabilities of both charge-separated states. Furthermore, since the increased stabilizations of $S_2Q_A^-$ and $S_2Q_B^-$ charge recombinations occur approximately in parallel, it seems likely that these changes are due to a modification of the H_2O-splitting enzyme specifically affecting the properties of the S_2 state and have negligible effects upon the properties of either Q_A^- or Q_B^-. The TL peak corresponding to the recombination of the $S_3Q_B^-$ charge pair also is upshifted in temperature in the mutant relative to the wild type (36 vs 24 °C, traces f and c, respectively). Since the stability of Q_B^- is largely unaffected by the absence of MSP, this indicates that the stability of the S_3 state is also increased by the absence of MSP.

Figure 5 shows the oscillation of the B band for the mutant and wild type as a function of the number of flashs given prior to recording the TL profile. In both the mutant and wild type, the intensity of the B band oscillates with a periodicity of four with the maximum emission occurring on the second flash. This is the same type of oscillation found for thermophilic cyanobacteria (Govindjee et al., 1985) and for whole leaf tissue (Rutherford et al., 1984) and provides additional evidence that the mechanistic characteristics of the H_2O-splitting enzyme of the mutant are similar to those of the wild type. The intensity of the thermoluminescence from the mutant sample is clearly much lower (37% on the first flash) than that obtained for the wild-type sample. It seems unlikely that the markedly reduced TL intensities seen for the mutant are due to solely to a greatly increased miss factor since, if that were the case, the oscillatory pattern would be much more highly damped than what is observed and maximum TL yield would be shifted to later flashes in the sequence. When the data are normalized to the first flash, the normalized TL yield for the mutant on the second flash is about 88% the normalized yield of the wild type. This value is similar to the difference between the mutant and wild type with respect miss factor deduced from the oscillations of flash O_2 yields using the bare platinum electrode as described above. Thus, it appears that the reduced TL intensities are due to a large extent to the existence of a large fraction of the PSII centers in the mutant which are inactive in oxygen evolution. It should be noted, however, that this interpretation assumes that the intrinsic luminescence yields from the recombination of charges in mutant reaction centers are equivalent to the corresponding yields from wild-type centers.

DISCUSSION

In this paper, we extend our investigation of photosystem II in a directed mutant of the cyanobacterium *Synechocystis* sp. PCC6803 in which the entire *psbO* gene encoding MSP has been deleted (Δ*psbO*). Under conditions of saturating continuous illumination and using DCBQ to accept electrons directly from the Q_B site of PSII, the mutant evolves oxygen at about $^1/_3$ the rate of the wild type [Burnap et al., (1991); Table I]. The lower maximal rates of oxygen evolution due to the absence of MSP could, in principal, be explained by several possibilities which are not mutually exclusive: (1) a reduction in the steady-state concentration of assembled reaction centers in the membranes of mutant cells; (2) an alteration of the acceptor side of the reaction center resulting in a reduced affinity for DCBQ; (3) a reduction in the proportion of assembled PSII reaction centers which are also competent in oxygen evolution; (4) a higher miss probability, e.g., reduced charge utilization efficiency in coupling the H_2O-splitting enzyme to reaction center turnover; and (5) a retardation in the rate(s) of one or more of the individual S-state transitions.

The results of [^{14}C]DCMU-binding assays (Figure 1 and Table I) and photochemical, DPC-supported DCPIP reduction measurements (Table I) indicate that the steady-state concentration of assembled, photochemically active PSII reaction centers is approximately the same in mutant and wild-type cells. From these results, we conclude that the reduced oxygen evolution activity is not due to a reduced concentration of assembled reaction centers in the membranes of Δ*psbO*. This conclusion is in reasonable agreement with the results of variable fluorescence yield measurements performed in the presence of hydroxylamine, which indicated that the mutant has greater than 80% of the functional reaction centers found in the wild type. It has been found previously that the mutant accumulates wild-type levels of all the intrinsic PSII proteins assayed immunologically (CP47, CP43, D1, and D2) (Burnap & Sherman, 1991; Philbrick et al., 1991). The present results suggest that the majority of these accumulated polypeptides are assembled into photochemically active complexes despite the absence of MSP.

The results of [^{14}C]DCMU-binding assays also indicate that the affinity of DCMU-binding sites is virtually unchanged by the deletion of the *psbO* gene. This argues against the occurrence of any dramatic alteration in the conformation of the Q_B site due to the lack of binding of MSP on the opposite side of the thylakoid membrane. It therefore seems less likely that the difference in oxygen evolution activity between the mutant and wild type using DCBQ as the electron acceptor is due to changes in the affinity for DCBQ on the acceptor side of PSII. Instead, it appears that the lower maximal rates of oxygen evolution are due to a limitation in the rate of electron transfer on the donor side of the reaction center. This accords well with the previous findings that the artificial electron donor

hydroxylamine restores the lower level of variable fluorescence observed for the mutant in the absence of an artificial electron donor to levels approaching that of the wild type (Burnap & Sherman, 1991; Philbrick, et al., 1991).

From the pattern of flash oxygen yield and thermoluminescence oscillation, we conclude that the mutant enzyme evolves oxygen by a mechanism similar to the wild type. However, the amplitudes of these signals are significantly reduced. Part of this reduction is due to an increase in the miss factor, α, as evidenced by the more highly damped oscillatory pattern of flash oxygen yield on the bare platinum electrode (Figure 2) exhibited by the mutant ($\alpha = 0.22$) compared to the wild type ($\alpha = 0.15$). The increase in the miss factor corresponds to an increased probability that oxygen-evolving reaction centers will fail to convert absorbed light quanta to productive advancements of the S system. The increase in the miss factor for MSP-less, but O_2-evolving centers, cannot, however, fully account for the extent in the reduction of the flash oxygen yield of the mutant, which is, at most, 40% of the wild type on the third flash (Figure 2). To account for the full extent of reduction in the flash O_2 signals, as well as the reduced TL intensities, we suspect that the mutant also has a substantial increase in the fraction of reaction centers which are completely inactive in O_2 evolution. This conclusion, was indirectly reached on the basis of fluorescence yield measurements (Philbrick et al., 1991). The centers inactive in O_2 evolution, but capable of mediating electron transport from DPC to DCPIP, may have lost active site manganese or be in a conformation incapable of catalyzing the H_2O-splitting reaction. It should be pointed out, however, that the reduced amplitudes of the flash oxygen and TL yields from the mutant may not be a simple function of a reduced number of oxygen-evolving centers but may also depend upon alterations in the kinetic properties of the mutant enzyme. In fact, preliminary measurements of steady-state flash O_2 yield (Kawamura et al., 1979; Myers et al., 1983) by the mutant and wild type suggest that the bare platinum flash O_2 yield and TL measurements described here may underestimate the relative proportion of O_2-evolving centers in the mutant (unpublished observations).

Assuming that the actual rate at which oxygen is released by the wild-type enzyme is in the order of 1–2 ms (Joliot et al., 1966; Etienne 1968; Babcock et al., 1976; Meunier & Popovic, 1991), the bare platinum electrode and conditions employed here cannot resolve this process kinetically (i.e., the observed rise of the wild-type signal is approximately 5 ms and would thus appear to be dominated by the diffusion time required for photosynthetic oxygen to reach the electrode surface). Since mutant and wild-type samples were treated identically (e.g., equivalent settling and dark-adaptation times), we make the assumption that the mean diffusion time for oxygen to reach the electrode following its release from the enzyme is approximately the same for the mutant and wild-type samples and about 5 ms. Since the observed exponential rise time of the oxygen signal from samples of the mutant is approximately 14 ms, we conclude that some dark step occurring during the O_2-yielding S_3–[S_4]–S_0 transition is slowed by at least a factor of 5 in the mutant enzyme compared to the wild-type enzyme. This conclusion is consistent with results of previous experiments using spinach PSII particles, which demonstrated that MSP accelerates the S_3–[S_4]–S_0 transition in vitro (Miyao et al., 1987).

One of the characteristics of $\Delta psbO$ is a greater susceptibility to photoinactivation (Philbrick et al., 1991; Mayes et al., 1991). At a high light intensities, the rate of oxygen evolution by the mutant cells declines much more rapidly in comparison to wild-type cells. In the context of present results suggesting that the mutant exhibits a higher miss factor and slowed kinetics of S-state advancement, it is expected that exposure of the mutant to high light intensities would result in the accumulation of oxidizing species (e.g., $P680^+$ and Y_Z^+) on the donor side of the reaction center due to the absence of efficient electron donation from the impaired H_2O-splitting enzyme. This situation might result in damage analogous to that observed for PSII membranes treated with hydroxylamine to extract active site Mn (Blubaugh et al., 1991). In this case, the loss of efficient electron donation to Y_Z appears to render PSII much more susceptible to photodamage to (or in the vicinity of) this redox-active amino acid residue.

Previous experiments examining the effects of removing MSP from PSII preparations in vitro by measuring thermoluminescence profiles (Vass et al., 1987) and by monitoring deactivation kinetics of the S states (Miyao et al., 1987) have indicated that the loss of MSP is accompanied by an increase in the stability of the S_2 state. In accordance with these findings, we observe approximately parallel increases in the stability of the $S_2Q_A^-$ and the $S_2Q_B^-$ charge recombinations in $\Delta psbO$ relative to the wild type, indicating that the loss of MSP is accompanied by a significant increase in the redox stability of the S_2 state. In the context of the present results, it is worth noting that previous thermoluminescence analysis of the S-state properties of PSII preparations (spinach BBY membranes) biochemically depleted of MSP (Vass et al., 1987) was complicated by apparent changes in the redox properties of the Q_B site (in addition to changes in the properties of the S_2 state). The TL peak for the $S_2Q_A^-$ charge recombination in MSP-depleted samples was shown to be markedly upshifted relative to the untreated control samples. On the other hand, the TL peak for the $S_2Q_B^-$ charge recombination in MSP-depleted samples was virtually unchanged relative to the control. It was tentatively concluded that the removal of MSP enhances the stability of the S_2 state, but the biochemical wash conditions (either 1.5 M $CaCl_2$ or 2.7 M urea/200 mM NaCl) used to remove MSP also cause a reduction in the stability of Q_B^- compensating the change in the stability of the S_2 state. In contrast to depletion experiments performed with spinach BBY membranes which were interpreted to show that Q_B is modified by depletion of MSP, the genetically constructed MSP-deletion mutant seems to retain normal properties of Q_B.

The TL peak for the recombination of the $S_3Q_B^-$ charge pair is also elevated in the mutant, suggesting that the S_3 state is also stabilized with respect to recombination with negative charges on the acceptor side of the reaction center. An enhancement in the stability of the S_3 state in the dark caused by the removal of MSP in vitro has also been reported, based upon its deactivation kinetics as monitored according to changes in oxygen yield as a function of the interval between the second and third flashes given to samples on a bare platinum electrode (Miyao et al., 1987). In contrast, the TL peak temperature for the recombination of the $S_3Q_B^-$ charge pair was not found to be upshifted in PSII preparations biochemically depleted of MSP (Vass et al., 1987). However, the absence of change in the thermal stability of the $S_3Q_B^-$ charge pair measured in $CaCl_2$- or urea/NaCl-washed PSII samples may again have been the result of changes in the stability of Q_B^- accompanying the removal of MSP, since the $S_3Q_A^-$ charge pair does exhibit a significant upshift in the TL peak temperature in PSII samples treated with 1.5 M $CaCl_2$ to remove MSP. It should be noted, however, that the

conclusion that the genetic loss of MSP results in an enhanced stability of the S_3 state is rendered more tentative in the absence of further information (e.g., thermal stability of the $S_3Q_A^-$ charge pair and the deactivation kinetics of the S_3 state) in mutant cells.

In conclusion, the functional consequences of the genetic removal of MSP in *Synechocystis* sp. PCC6803 are quite complex. Although the number of photochemically active PSII reaction centers is not much changed by the absence of MSP, the proportion of centers which are coupled to functional O_2-evolving enzymes appears to be significantly reduced. For those centers which are effectively coupled to O_2 evolution, we find evidence of alterations in the kinetic properties of the enzyme due to the absence of MSP. These are (1) an increased miss factor; (2) a retardation in one of the dark steps of the catalytic cycle, the S_3–$[S_4]$–S_0 transition, and (3) an increase in the stabilization of the S_2 and S_3 states. Since, the absence of MSP appears to result in an increase in the stabilization of the S_3 state, we speculate that the observed slowing of the S_3–$[S_4]$–S_0 transition is due to the mutant enzyme's reduced ability to overcome the activation energy required for passage through this O_2-yielding transition. This scenerio would account for the increased miss factor since a greater fraction of centers would fail to advance on the succeeding flash. While deactivations can also contribute to misses, S_2 and S_3 are more stable, and it is doubtful that deactivations (at least from these states) account for the observed increase in miss probability. Instead it seems more likely that the increased chance for missed advancement of the enzyme by a flash is directly due an alteration in the forward kinetics of S-state advancement in the absence of MSP.

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