Role of Disulfide Linkage and Putative Intermolecular Binding Residues in the Stability and Binding of the Extrinsic Manganese-Stabilizing Protein to the Photosystem II Reaction Center†

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ABSTRACT: Site-directed mutations were produced at three highly conserved amino acid positions of MSP of the photosystem II (PSII) reaction center in the transformable cyanobacterium *Synechocystis* sp. PCC6803. The highly conserved aspartate at position 9 of the mature MSP was changed to a lysine (strain MSP-D9K) to assess its role in the proposed N-terminal binding region (Eaton-Rye & Murata, 1989; Odom & Bricker, 1992). This strain accumulates normal levels of MSP, and the properties of the H_2O-splitting enzyme are only slightly altered relative to the wild-type. In contrast, replacement of cysteine 20 with a serine, which is proposed to disrupt the intramolecular disulfide bridge (Tanaka et al., 1989), produces a phenotype with no detectable accumulation of MSP, despite normal levels of mRNA transcripts. Like the *psbO* deletion strain, the MSP-C20S mutant exhibits impaired O_2 evolution activity and a greater stability of the S_2 state as measured by thermoluminescence. Mutation of strictly conserved aspartate 159 to asparagine (MSP-D159N) does not affect the accumulation of MSP, but causes a reduction in the H_2O-oxidation activity and thermoluminescence properties intermediate between the wild-type and the *psbO* deletion strain. In addition, we report upon improved methods for obtaining oxygen-evolving membranes from mutant cells.

The membrane bound photosystem II (PSII)[1] complex catalyzes the light driven transport of electrons from water to plastoquinone in a process that forms the basis for oxygenic photosynthesis. The mechanism of the H_2O-splitting reaction and the structure of the metalloprotein catalyzing this reaction remain to be clarified. A cluster of four Mn atoms is responsible for the accumulation of at least some of the strong oxidizing equivalents generated during each of the four turnovers of the photochemical reaction center required for the oxidation of substrate H_2O. The accumulation of oxidizing equivalents corresponds to the successive extraction of electrons from the metalloprotein domain of the H_2O-oxidation complex and gives rise to a series of five oxidation states (S-states), termed S_0−S_4, of the complex. The four-electron oxidation of the two substrate H_2O molecules completes the catalytic cycle and restores the H_2O-oxidation complex to the S_0 state.

At least five major intrinsic membrane proteins, CP47, CP43, D1, D2, and cytochrome *b*-559, comprise the PSII reaction center complex. Additionally, several low molecular weight polypeptides have been identified as components of the intrinsic complex, and chloride and calcium are thought to be the inorganic cofactors involved in the formation of the enzymatically active complex. None of the aforementioned polypeptides has been experimentally excluded as participating in the ligation of the active site Mn. However, several lines of evidence support the hypothesis that the D1 protein supplies a number of the ligands to active site Mn (Boerner et al., 1992; Chu et al., 1994; Ikeuchi et al., 1988; Nixon & Diner, 1992; Nixon et al., 1992; Seibert et al., 1989).

The manganese-stabilizing protein (MSP) is a strongly-bound, extrinsic polypeptide associated with the lumenal portion of the photosystem II (PSII) complex where the photosynthetic oxidation of water is catalyzed. Biochemical reconstitution experiments have shown that MSP stabilizes the binding of 2 of the 4 Mn atoms present at the catalytic center of the H_2O-splitting enzyme (Ono & Inoue, 1984), although high concentrations (>200 mM) of Cl^- can substitute for MSP in this regard (Kuwabara et al., 1985). Biochemical and molecular genetic studies have indicated that MSP modulates the activity of the H_2O-splitting reaction and its calcium requirement. Molecular genetic studies of cyanobacterial strains lacking MSP have corroborated earlier biochemical data suggesting that MSP is not absolutely essential for functioning of the H_2O-splitting enzyme (Bock-

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[1] Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, inhibits electron transport between Q_A and Q_B; Km^r, resistant to the antibiotic kanamycin; MSP, manganese-stabilizing protein, extrinsic 33 kDa PSII protein; Q_A, primary plastoquinone electron acceptor; Q_B, secondary, exchangeable plastoquinone electron acceptor; 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; Sp^r, resistant to the antibiotic spectinomycin; TL, thermoluminescence.

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holt et al., 1991; Burnap & Sherman, 1991; Mayes et al., 1991; Philbrick et al., 1991). However, mutants lacking MSP exhibit markedly altered kinetic properties (Burnap & Sherman, 1991; Burnap et al., 1992a; Philbrick et al., 1991; Vass et al., 1992) and an increased susceptibility to photoinhibition (Mayes et al., 1991).

Based upon a variety of experimental results, the binding of MSP to the intrinsic portion of the PSII complex has been proposed to involve ionic interactions as well as extensive hydrogen bonding (Enami et al., 1991; Miyao & Murata, 1989; Odom & Bricker, 1992; Shen & Inoue, 1991). MSP has been proposed to physically contact with, or be in close proximity to, virtually all of the intrinsic PSII proteins. The strongest evidence for a specific interaction between MSP and individual PSII intrinsic polypeptide comes from immunological and chemical cross-linking studies, which indicate a direct contact between CP47 and MSP that is probably mediated via salt-bridge interactions between the two proteins (Bricker et al., 1988; Enami et al., 1991; Frankel & Bricker, 1992; Odom & Bricker, 1992). Proteolytic cleavage of first 18−20 N-terminal amino acids of the mature MSP prevents binding to the reaction center despite the fact that the tertiary structure of the protein does not appear to be altered as assayed by circular dichroism (Eaton-Rye & Murata, 1989). These results are regarded as evidence for the importance of the N-terminal region of MSP for binding. Reductive cleavage of the sole disulfide bridge in MSP results in the unfolding of the protein and the concomitant inability of the protein to bind to the reaction center (Tanaka et al., 1989), indicating the importance of the intramolecular linkage for maintenance of tertiary structure and the necessity for native MSP structure for binding. Here we report the characteristics of cells expressing mutant MSPs in which site-directed mutations were produced at highly conserved amino acid positions that were proposed to influence the binding of the protein to the lumenal portion of the PSII reaction center. Preliminary accounts of this work were presented at the International Congress on Photosynthesis (Burnap et al., 1992b).

MATERIALS AND METHODS

Directed Mutagenesis of the psbO Gene. The naturally transformable, glucose-utilizing strain of *Synechocystis* sp. PCC6803 (Williams, 1988) was used in the construction of all of the strains described here. For routine manipulations, the wild-type and mutant cyanobacterial strains were grown on a rotary shaker in BG-11 media supplemented with 5 mM glucose at 32 °C. The construction of the strain designated ΔpsbO which lacks the entire *psbO* coding sequence has been described previously (Burnap & Sherman, 1991). This strain was utilized as a recipient of mutant *psbO* alleles having defined amino acid substitutions within the MSP coding sequence yielding strains which express mutant forms of MSP. The overall strategy used to introduce the desired mutations into the deletion strain ΔpsbO involved transformation with a mutagenic plasmid containing mutant alleles of the *psbO* gene. The mutagenic plasmids were derivatives of plasmid pRB81 with oligonucleonucleotide-directed mutations constructed within the *psbO* coding sequence. The plasmid pRB81 contains the full-length wild-type *psbO* gene flanked by an antibiotic resistance gene conferring kanamycin resistance (Km^r). pRB81 was constructed by ligating the resistance cassette, derived from Tn903, into an *Xba*I restriction site approximately 400 bp downstream of the *psbO* coding sequence in the plasmid pRB1. Plasmid pRB1, described in Burnap and Sherman (1991), has a 1.9 kb chromosomal fragment of *Synechocystis* sp. PCC6803 containing the *psbO* gene cloned into the Bluescript phagmid vector KS(−) (Stratagene Inc., La Jolla, CA).

Site-specific mutations were introduced into the cloned *psbO* gene essentially according to the Kunkel method (Kunkel, 1985; McClary & Witney, 1991). The entire *psbO* operon region (about 1000 base pairs) was sequenced to ensure that additional mutations were not inadvertently introduced. Stable introduction of mutant *psbO* genes into strain ΔpsbO was accomplished with transformation procedures (Burnap & Sherman, 1991; Williams, 1988) which rely on homologous recombination of exogenous DNA sequences into the recipient chromosome. Direct selection of recombinants depended upon the flanking Km^r cassette which is inserted in a nonessential, nontranscribed downstream region such that it acts as a selectable marker, but does not otherwise affect any observable functions (including expression of MSP) when transformed into *Synechocystis*. Chromosomal DNA isolated from the transformants was analyzed by Southern blot procedures to confirm that the predicted recombinational events had occurred. Genotypes were further analyzed at the DNA sequence level by direct sequence analysis of polymerase chain reaction (PCR) products, which were amplified from chromosomal DNA isolated from the mutants.

Isolation of Oxygen-Evolving Membranes. Isolation of oxygen-evolving membranes was performed using modifications of previously described procedures (Burnap et al., 1989). Cultures were grown in flattened tissue culture bottles with 5% CO_2 enriched air under white incandescent illumination [100 $\mu E/(m^2 \cdot s)$]. Rapid chilling of cultures was found to greatly improve the retention activities of the ΔpsbO and MSP-C20S strains which otherwise decayed with a half-time of 10−20 min in the dark. Cells harvested from 700 mL late logarithmic phase cultures were resuspended in approximately 5 mL of HMCS buffer (50 mM Hepes−NaOH, pH 7.2, 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 1.0 M sucrose) or HMCS containing 500 mM NaCl (HMCS-HS) and placed on ice 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 6 mL aliquot of the resuspended cells was transferred to a 12 mL Braun homogenizer tube, and glass beads (0.1 mm) were added to the tube such that a small airspace (about 0.5 mL) remained once the tube was closed. DNase (Sigma Chemical Co.) was added to a concentration of 100 μg/mL. The sample was then subjected to 5 min of agitation at top speed (about 3000 rpm) in a Braun homogenizer under a cooling stream of CO_2 gas from a siphon tank. The flow rate of the cooling stream was carefully adjusted to ensure that the sample temperature remained close to 0 °C during the entire breakage period. Glass beads, unbroken cells, and debris were removed from the cell homogenate by centrifugation at 5000 rpm in a Sorvall SS-34 rotor. After recovery of the resultant supernatant, the glass beads were extracted two to three times with fresh buffer and pelleted as before to recover trapped thylakoids. Membranes were pelleted from the combined supernatants by centrifugation at 150000*g*. in a benchtop ultracentrifuge for 30 min. At this stage, membranes isolated from both wild-type and ΔpsbO cells retain

a significant fraction (>80%) of the DCBQ-supported, O_2 evolution activity present in the cells used as starting material.

Northern and Immunoblot Analysis. Cells used for Northern blot and immunoblot analysis were obtained from late-log phase cultures grown in the presence of 5 mM glucose. Isolation of total cellular RNA and subsequent Northern blot analysis and immunoblot analysis of whole cell lysates were performed as described previously (Burnap & Sherman, 1991).

Oxygen Evolution and Other Assays. Oxygen evolution under continuous illumination and thermoluminescence measurements were performed essentially as described previously (Burnap & Sherman, 1991; Burnap et al., 1992a). One point that proved to be critical for the maintenance of whole cell oxygen evolution activity in certain mutants was to maintain cells in the light on a rotary shaker during the interval between post-harvest resuspension and assays. Measurement of liquid nitrogen temperature fluorescence emission spectra was performed as described in Burnap and Sherman (1991).

RESULTS

Directed Mutagenesis of the psbO Gene. We have constructed three mutant strains of *Synechocystis* sp. PCC6803 with defined single amino acid substitutions within the mature portion of the MSP sequence. These mutations were produced at conserved positions within the MSP sequence by oligonucleotide-directed mutagenesis of the cloned *psbO* gene present in the transformation plasmid, pRB81. Plasmid pRB81 was constructed such that homologous double crossover recombination (the predominant recombinational mode in *Synechocystis* sp. PCC6803) into the Δ*psbO* chromosome would occur in a manner which restores the *psbO* gene (absent in the recipient) and at the same time render the transformant resistant to kanamycin. In practice, all kanamycin resistant transformants isolated thus far have proven to occur via this mechanism as ascertained by Southern blot and sequence analysis.

Based on published reports (Eaton-Rye & Murata, 1989; Odom & Bricker, 1992), two of the mutations were constructed to alter the binding of MSP to the reaction center. The first 18 amino acids of the NH_2-terminal portion of the spinach MSP were shown to be required for the binding of MSP to the PSII reaction center *in vitro* (Eaton-Rye & Murata, 1989). Charged residues within the N-terminal 70 amino acids of MSP are believed to interact with the putative lumenal ("E") loop of CP47 (Odom & Bricker, 1992). Asp-9 is the most highly conserved charged residue in the portion of the determined by Eaton-Rye and Murata to be important for binding and was targeted as a potential member of a charge pair mediating the binding of MSP to the reaction center. Chemical reduction of the intramolecular disulfide bridge in MSP has been shown to prevent binding *in vitro* of the protein to the reaction center (Tanaka et al., 1989). Cys-20 is postulated to form an intramolecular disulfide bridge with strictly conserved Cys-45, the only other cysteine occurring in the mature *Synechocystis* protein (Philbrick & Zilinskas, 1988). To disrupt this disulfide linkage genetically, we replaced the strictly conserved cysteine located at position 20 (Cys-20) of the mature *Synechocystis* MSP sequence with a serine. The third strain, MSP-D159N, involves a substitution of a strictly conserved aspartate located in one of the most highly conserved charged regions in MSP and was targeted as a potential member of a charge pair involved in MSP binding or as a potential ligand to Ca^{2+}, since MSP is known to modulate the Ca^{2+} requirement of PSII activity in *Synechocystis* (Philbrick et al., 1991) and in spinach membranes (Bricker, 1992).

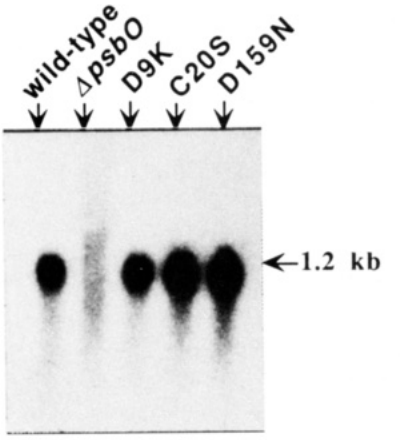

FIGURE 1: Northern blot analysis of the *psbO* transcript in wild-type, ΔpsbO, MSP-D9K, MSP-C20S, and MSP-D159N cells. Ten-microgram aliquots of total cellular RNA were electrophoresed on a 1.2% agarose gel containing formaldehyde and blotted onto Nytran membranes (Schleicher & Scheull Inc.). The filter immobilized RNA was hybridized with a [α-^{32}P]dCTP-labeled restriction fragment containing the *psbO* coding sequence from *Synechocystis* sp. PCC6803. The resultant radioactive hybrids were visualized by autoradiography.

Expression of the Mutant Genes. The three amino acid substitution mutant strains were examined to determine the expression of the mutated *psbO* alleles. Analysis of the messenger RNA levels for the *psbO* gene is shown in the Northern blot (Figure 1). The *psbO* transcript in the wild-type is estimated to be approximately 1.2 kilobases in length, as shown previously (Burnap & Sherman, 1991; Mayes et al., 1991). The absence of *psbO* transcripts in ΔpsbO is expected since the complete *psbO* coding sequence has been deleted from the chromosome in this strain (Burnap & Sherman, 1991). On the other hand, transformation of the ΔpsbO strain with the site-directed mutant derivatives of pRB81 results in the restoration of the *psbO* transcripts in each of the transformants examined. Furthermore, each site-directed mutant strain accumulates wild-type levels of the *psbO* transcript, indicating that transcription of the *psbO* gene is not modified by the genetic alterations produced during construction.

The results of immunoblot analysis using anti-spinach MSP antibody to probe whole cell lysates of each strains are shown in Figure 2. An immunoreactive band is observed for the wild-type, but is absent in ΔpsbO, as expected from previous results (Burnap & Sherman, 1991). An accumulation of wild-type levels of MSP is observed in the MSP-D9K and MSP-D159N mutants, suggesting that amino acid substitutions at these positions do not ostensibly affect the expression and stability of MSP nor do they perturb reactivity of the mutant protein with the antibody. In contrast, the MSP-C20S mutant, which is unable to form the normal intramolecular disulfide bridge, does not accumulate detectable amounts of MSP, despite the accumulation of normal levels of mRNA transcript (Figure 1).

Oxygen Evolution by Whole Cells and Membranes. Maximal rates of oxygen evolution by whole cells were

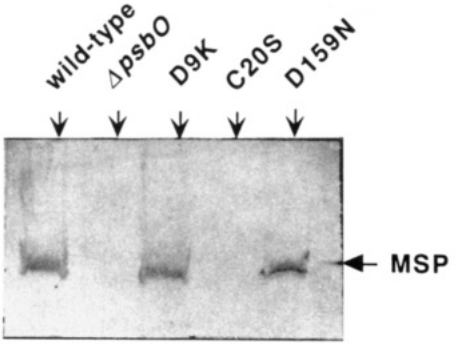

FIGURE 2: Immunoblot analysis of whole cell lysates using antibody reacting with MSP. Samples containing 5 μg of chlorophyll were denatured in the presence of SDS and mercaptoethanol and electrophoresed on a 12% polyacrylamide gel, then transferred to nitrocellulose, and reacted with antibody directed against spinach MSP.

Table 1: Oxygen Evolution[a] by Whole Cells and Isolated Thylakoids

	whole cells[a] (% wild-type)	membranes[b,c] (% wild-type)
wild-type	100 ± 14 (8)	100 ± 5 (5)[b]
ΔpsbO	39 ± 8 (7)	36 ± 5 (5)[c]
MSP-D9K	90 ± 15 (4)	82 ± 11 (4)[b]
MSP-C20S	42 ± 7 (4)	36 ± 5 (4)[c]
MSP-D159N	64 ± 11 (4)	44 ± 2 (4)[b]

[a] Maximal rates of O_2 evolution [μmol of O_2·(mg of Ch1)$^{-1}$·h^{-1}] under continuous saturating illumination was measured at 30 °C with the addition of 600 μM DCBQ and 5 mM $K_3Fe(CN)_6$, and with samples at a concentration of 7.5 ′g of chlorophyll-mL^{-1}. Wild-type cells exhibited an average rate of 465 μmol of ($_2$·(mg of Ch1)$^{-1}$·h^{-1}. Values are expressed as averages with the corresponding standard deviation obtained from a set of n measurements (values in parentheses = 4n). [b] Membranes isolated and assayed in HMCS buffer (50 mM Hepes−NaOH, pH 7.2, 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 1.0 M sucrose). Values in parentheses = n. [c] High-salt membranes: membranes isolated and assayed in HMCS buffer supplemented with 500 mM NaCl.

estimated using 2,6-dichlorobenzoquinone (DCBQ) and potassium ferricyanide as an electron acceptor system (Table 1). The *psbO* deletion mutant exhibits a markedly reduced (30−40%) rate of oxygen evolution relative to the wild-type despite the accumulation of nearly wild-type levels of assembled PSII reaction centers in the absence of MSP (Burnap & Sherman, 1991; Burnap et al., 1992a). It is therefore not surprising that MSP-C20S exhibits oxygen evolution characteristics closely resembling those of ΔpsbO, since MSP-C20S does not accumulate MSP (Figure 2). In contrast, strain MSP-D9K exhibits whole cell rates that are only slightly impaired relative to the wild-type. On the other hand, strain MSP-D159N exhibits significantly reduced (60−65%) maximal rates of oxygen evolution relative to the wild-type, despite the fact that it accumulates wild-type levels of MSP.

Table 1 also shows the rates of oxygen evolution for thylakoid membranes isolated from each of the strains. MSP-D9K proved amenable to previously developed membrane isolation procedures (Burnap et al., 1989) for isolating oxygen-evolving membranes from wild-type *Synechocystis* sp. PCC6803 cells, although the activity was more labile than the wild-type as judged by an increased tendency to lose activity during membrane isolation. In contrast, the strain MSP-C20S was not amenable to our standard membrane isolation procedure, as found previously for ΔpsbO (Burnap

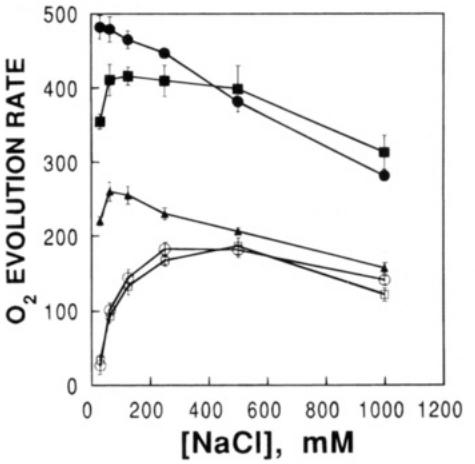

FIGURE 3: Maximal oxygen evolution rates by membranes isolated from *Synechocystis* sp. PCC6803 wild-type and site-directed mutants of *psbO* as a function of NaCl concentration. Assay buffer consisted of HMCS buffer (50 mM Hepes−NaOH, pH 7.2, 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 1.0 M sucrose) adjusted to the indicated concentrations of NaCl. Wild-type (●); D9K (■); C20S (○); ΔpsbO (□); D159N (▲).

& Sherman, 1991). Because high chloride concentrations are known to stabilize the active site Mn in the absence of MSP (Kuwabara et al., 1985), we modified the standard buffer of our procedure (HMCS) to include high NaCl concentrations (HMCS-HS, see Materials and Methods). Under these conditions, good retention of the pre-cell breakage oxygen evolution activity (≥80%) was obtained. Furthermore, the activity of membranes extracted in this manner was very stable, losing very little activity during 8 h on ice. Purification of oxygen-evolving membranes from MSP-D159N was the most problematic of the mutant strains, since substantial declines in activity were observed for all conditions employed here. Highest MSP-D159N membrane activities (65% of the already depressed whole cell rates) were obtained with low salt buffer conditions. Extraction of membranes under high salt conditions resulted in a large initial decline in activity and yielded a final preparation that was virtually indistinguishable from high-salt ΔpsbO and MSP-C20S membranes in terms of activity and stability (data not shown).

The dependence on NaCl concentration of oxygen evolution activity of membranes from different strains was further investigated as documented in Figure 3. In these experiments, membranes were assayed in reaction buffers containing different concentrations of NaCl. For both ΔpsbO and MSP-C20S, the rate of oxygen evolution increased with increasing concentrations of NaCl, reached a maximum at approximately 500 mM NaCl, and decreased at higher concentrations. Membranes isolated from wild-type cells, on the other hand, exhibited a steady decrease in activity with increasing NaCl concentration. In contrast to these trends, membranes from MSP-D159N and MSP-D9K cells exhibited clear and reproducible maxima at intermediate concentrations of NaCl. The existence of these maxima may reflect heterogeneity of PSII centers in the MSP-D159N and MSP-D9K thylakoid preparations with respect to the presence of bound MSP. The fraction of centers that do not have bound MSP would be stable only at elevated salt concentrations, whereas the remainder of centers with bound MSP would be most active at low salt concentrations. In this situation, oxygen evolution activity would be the sum of rates

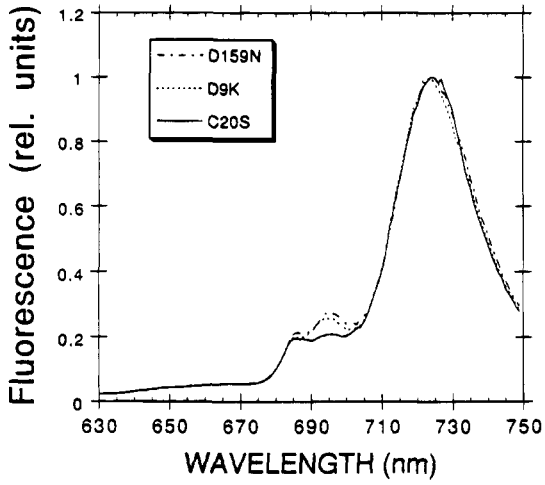

FIGURE 4: Fluorescence emission spectra of whole cells under excitation of chlorophyll (435 ± 5 nanometers) recorded at 77 K.

of two distinct populations of centers, each responding to salt concentration in an opposite manner, and, in aggregate, give rise to a total maximal activity at some intermediate salt concentration.

Low Temperature Fluorescence. The fluorescence emission at 77 K of whole cells excited by light absorbed primarily by chlorophyll is a sensitive indicator of the state of chlorophyll in photosynthetic membranes (Murata & Satoh, 1986). Emissions at 685 and 696 nm are ascribed to pigments associated with photosystem II, whereas the emission centered at approximately 725 nm can be traced to pigments associated with PSI. A previous study comparing the fluorescence characteristics of the wild-type and *psbO* deletion mutant showed that removal of MSP resulted in a reduction of the emission centered at 696 nm (Burnap & Sherman, 1991). The 77 K emission spectra of MSP-D9K, MSP-C20S, and MSP-D159N are shown in Figure 4. MSP-C20S is virtually identical to the *psbO* deletion mutant, whereas strains MSP-D9K and MSP-D159N, which accumulate wild-type levels of MSP, exhibit rather normal spectra compared with that of the wild-type (wild-type spectrum not shown).

Thermoluminescence. Figure 5 shows the thermoluminescence (TL) profiles obtained for each of the mutants with and without the addition of the electron transport inhibitor DCMU. These measurements provide information on the depth of the activation energy wells involved in the stabilization of the $S_2Q_A^-$ and $S_2Q_B^-$ charge separated states (DeVault & Govindjee, 1990). Single turnover flash illumination followed by rapid cooling in liquid N_2 was used to generate and trap the charge separated states. The predominant charge separated states in these experiments are the $S_2Q_A^-$ (with DCMU) and $S_2Q_B^-$ (without DCMU). Different charge separated states exhibit different characteristic temperatures at which the detrapping of separated charges occurs. For example, the $S_2Q_A^-$ recombination in PSII membrane fragments from spinach typically peaks at between 5 and 10 °C, whereas the $S_2Q_B^-$ recombination typically occurs around 30 °C.

As shown previously, both the $S_2Q_A^-$ and $S_2Q_B^-$ charge recombinations in the ΔpsbO strain (Figure 5) are upshifted in peak temperatures compared to those of the wild-type (Burnap et al., 1992a). These results were interpreted as reflecting an alteration on the donor side of PSII affecting

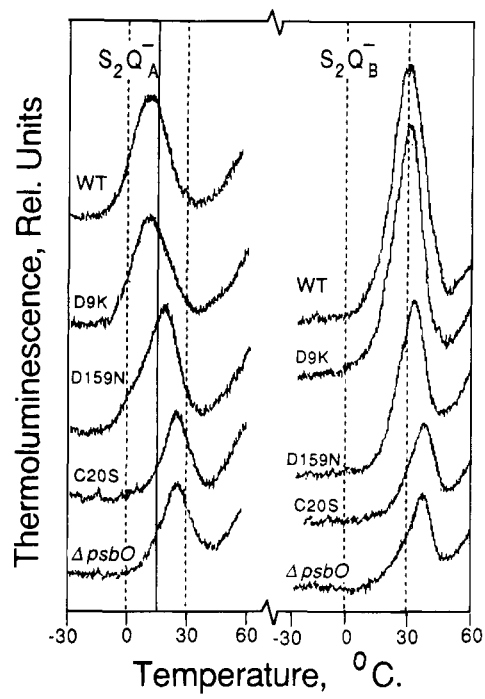

FIGURE 5: Thermoluminescence emission profiles of whole cells in the presence (left, $S_2Q_A^-$) and absence (right, $S_2Q_B^-$) of DCMU. Cells were dark adapted on ice for 10−15 min, in the presence or absence of 20 μM DCMU, prior to flash illumination, which was given at 0 °C. Samples were warmed at a rate of approximately 2 °C/min, during which time luminescence was recorded.

Table 2: Thermoluminescence Properties of *Synechocystis sp.* PCC6803 and Various Mutants of the *psbO* gene

	thermoluminescence peak temp (°C)			
	$S_2Q_A^-$	$S_2Q_B^-$	F696	MSP
wild-type	11	30	normal	present
ΔpsbO	25	38	reduced	absent
MSP-D9K	10	30	normal	present
MSP-C20S	25	38	reduced	absent
MSP-D159N	17	33	normal	present

the charge transfer characteristics of the S_2 state due to the absence of MSP [cf Vass et al. (1992) and see Discussion]. Virtually identical changes in the TL profiles of both the $S_2Q_A^-$ and $S_2Q_B^-$ charge recombinations were observed for the MSP-C20S strain (Figure 5) as might be expected based on the absence of MSP accumulation in this strain. In both the ΔpsbO and MSP-C20S strains, the peak temperatures of the $S_2Q_A^-$ and $S_2Q_B^-$ charge recombinations were upshifted by 14 and 8 °C, respectively, and correspond to enhanced stabilities of these charge separated states relative to the wild-type (Table 2). The peak temperatures of the $S_2Q_A^-$ and $S_2Q_B^-$ charge recombinations for MSP-D159N were both upshifted by 7 and 4 °C, respectively, compared to the wild-type. On the other hand, the TL properties for the MSP-D9K strain were quite similar to those of the wild-type, although the peak temperatures for both of the measured recombinations were actually slightly lower than those of the wild-type.

DISCUSSION

Previous investigations of the function of MSP in *Synechocystis* sp. PCC6803 have determined that MSP is required for optimal function of the H_2O-oxidation complex,

but that H_2O-splitting activity exists, albeit impaired, in its absence (Burnap & Sherman, 1991; Burnap et al., 1992a; Mayes et al., 1991; Philbrick et al., 1991; Vass et al., 1992). This paper documents the first site-directed mutagenesis investigation of conserved amino acids of MSP in *Synechocystis* sp. PCC6803. We have shown the following: (1) The phenotype of MSP-C20S is very similar to that of ΔpsbO, and we propose that MSP exists in an unfolded state in this mutant, thus rendering the protein susceptible to proteolytic degradation; (2) MSP-D9K does not have a dramatic effect on O_2 evolution, which suggests either that D9 does not form a salt bridge to the reaction center or that this bridge is not critical; (3) MSP-D159N significantly influences the rate and stability of O_2 evolution, the thermoluminescence properties of the H_2O-splitting enzyme, and the binding affinity of the protein for the PSII reaction center. Recently, Seidler and co-workers (1992) also have reported on the construction of amino acid substitution mutants of spinach MSP and the reconstitution of the molecules with polypeptide depleted spinach membranes.

One of the more striking results of this study is the lack of accumulation of MSP following the genetic disruption of the intramolecular disulfide linkage postulated to exist between C20 and C45. In contrast, the other two strains with single amino acid substitution mutations (MSP-D9K and MSP-D159N) accumulate wild-type levels of MSP. Although several possibilities could account for the lack of accumulation of MSP in MSP-C20S, it seems likely that MSP is prone to proteolytic degradation in the absence of an intramolecular disulfide linkage. Indeed, the chemical cleavage of the disulfide linkage causes the unfolding of the protein and prevents its binding to the reaction center *in vitro* (Tanaka et al., 1989). Therefore, the mutant MSP may exist in an unfolded and unbound state in the cell, which is proposed to increase susceptibility of the mutant protein to proteolytic cleavage. Alternatively, the lack of the disulfide bridge may somehow hinder the processing and/or transport of the nascent protein across the thylakoid membrane into the lumen, again causing it to be prone to degradation. Recently, a stoichiometry of two MSP molecules per reaction center was reported (Xu & Bricker, 1992), raising the possibility of a dimeric configuration of MSP stabilized by *inter*molecular disulfide linkage(s). This putative linkage might then contribute to the metabolic stability of MSP. However, the existence of such intermolecular linkage(s) seems unlikely given sedimentation velocity experiments indicating a monomeric native species (Miyao & Murata, 1989) and electrophoretic mobility/immunoblot experiments comparing SDS-solublized membranes in the presence and absence of sulfhydryl reducing agents which show only small changes in mobility (Burnap, unpublished).

Earlier attempts at isolating oxygen-evolving membranes from ΔpsbO proved unsuccessful due to the extreme lability of oxygen evolution activity in the absence of MSP (Burnap & Sherman, 1991; Burnap et al., 1992a). The loss of activity in ΔpsbO and MSP-C20S could be largely prevented by including 0.5 M NaCl in the standard cell breakage buffer and careful control of temperature during cell breakage (Table 1). Although the ion specificity for the stabilization of oxygen evolution activity in ΔpsbO and MSP-C20S has not yet been investigated, it seems likely that Cl^- is responsible for the stabilizing effect. This conclusion is based on the analogous experiments performed using MSP-extracted PSII preparations from spinach (Kuwabara et al., 1985).

The D159N mutation had a significant impact upon the rate and stability of O_2 evolution (Table 1), despite the fact that normal amounts of MSP were accumulated. The instability of MSP-D159N was particularly evident during the isolation of membranes, where its activity proved to be even more labile than that of MSP-D9K. Large declines in activity were invariably observed during the initial stages of membrane isolation, especially with high salt in the isolation buffer. This suggests that the D159N mutation significantly alters the binding affinity of MSP for the reaction center. In contrast to our results, Seidler et al. (1992) observed only a minor impairment of O_2 evolution with the D157N mutation in spinach MSP (equivalent to the D159N mutation in *Synechocystis*). One possible explanation for this discrepancy is that D159N mutation in *Synechocystis* affects important intermolecular interactions that are different or simply not present in spinach PSII complex. Possible candidates for this putative intermolecular interaction could include low molecular weight polypeptides which appear to be unique to the cyanobacterial H_2O-oxidation complex. These may include a *Synechocystis* homologue to the 9−12 kDa polypeptide found in thermophilic cyanobacteria, which has been shown to be necessary for maximal activity (Stewart et al., 1985; Shen & Inoue, 1993). Additionally, a low potential *c*-type cytochrome, designated *c*-550, appears to be in close association with MSP and is required for maximal activity (Shen & Inoue, 1993).

The minimal impact of the D9K mutation on O_2 evolution was somewhat surprising, in that the aspartate 9 residue is so highly conserved and is situated in the region identified as critical for the binding of MSP to the reaction center (Eaton-Rye & Murata, 1989). A similar result was obtained by Seidler et al. (1992) using their spinach *in vitro* reconstitution system. These results suggest that aspartate 9 is not involved in a charge-pair interaction with the lumenal portion of an intrinsic reaction center polypeptide as initially hypothesized. Although ionic interactions are predicted to exist as part of the binding of MSP to the reaction center, they may involve charged residues elsewhere on MSP that are consistent with chemical cross-linking studies (Odom & Bricker, 1992). The cross-linking data indicate that charge-pair interactions are likely to exist between the large (about 190 amino acid) lumenal E-loop of CP47 and the N-terminal 70 amino acids of MSP.

The 77 K fluorescence emission characteristics of the various mutants show a clear parallel between the presence of MSP and the intensity of the 696 nm emission (Figure 4). It was observed previously that deletion of the *psbO* gene, and the consequent absence of MSP, resulted in a decrease in the intensity of the 696 nm fluorescence emission of the 77 K spectrum (Burnap & Sherman, 1991). The 696 nm emission has been tentatively assigned to chlorophylls associated with CP47, although the amount of immunologically detectable CP47 does not change as a result of the deletion of the *psbO* gene. Therefore, we conclude that the binding of MSP to the intrinsic portion of the reaction center modulates the fluorescence yield of the F696 emitting chlorophyll(s) through a conformational change in CP47.

The peak temperature of thermoluminescence emission due to the recombination of charge pairs generated by the

excitation of PSII depends, according to theory, upon the total free energy of detrapping. This includes the redox potential gap between the recombining charge pair and the activation energy for the rate-limiting step of charge recombination. Previously, we observed that genetic removal of MSP (the *psbO* deletion strain) resulted in concurrent upshifts in the peak temperatures for the $S_2Q_A^-$ and for the $S_2Q_B^-$ charge recombinations (Burnap et al., 1992a). This was interpreted as being due to an increase in the stability of the S_2 state toward recombination. The present results show that MSP-C20S and MSP-D159N also exhibit upshifts in TL peak temperatures for both the $S_2Q_A^-$ and $S_2Q_B^-$ charge pair recombinations, indicating increased stability of the S_2 state. As before, however, the upshifts in temperature for the $S_2Q_A^-$ and $S_2Q_B^-$ are not equal in magnitude (e.g., 14 and 8 °C, respectively, for MSP-C20S). Does this mean that *psbO* mutations also alter the acceptor side of PSII affecting the stability characteristics of Q_B^-? In principal, alterations in the acceptor side of PSII could be caused by structural perturbations on the lumenal portion of the PSII complex that are transmitted allosterically across the membrane. Although our data cannot answer this question directly, the possibility that *psbO* mutations can induce structural changes in the intrinsic portion of PSII seems reasonable considering the changes in the F696 signal discussed above. Vass et al. (1992) conclude that genetic removal of MSP alters the acceptor side of PSII based upon their TL measurements of their *psbO* deletion mutant, designated IC2. However, in contrast to the TL results reported here, they do not observe concurrent upshifts of the peak temperatures $S_2Q_A^-$ and $S_2Q_B^-$ charge pair recombinations. Instead, they observe a 12 °C upshift for the $S_2Q_A^-$ recombination, similar to our results, but they report a 5 °C downshift for the $S_2Q_B^-$ recombination. It is not clear what accounts for this discrepancy, but different sample preparation protocols make a direct comparison difficult. In our protocol, samples were chilled on ice during the dark adaptation (relaxation of the H_2O-oxidation complex to primarily the S_1 state) prior to the actinic flash, which was found to minimize otherwise pronounced variations in the $S_2Q_B^-$ recombination peak temperature. Using a similar chilling/dark adaptation protocol, Carpenter et al. (1993) reported a wild-type $S_2Q_B^-$ recombination centered around 33 °C. On the other hand, Vass et al. (1992) utilized a 30 s illumination period prior to dark adaptation and obtained a $S_2Q_B^-$ recombination peak temperature of around 40 °C for the wild-type. While an understanding of these differences will require further analysis, it is known that certain experimental parameters, such as differences in heating rate, affect TL peak temperatures. Additionally, preliminary results measuring S_2 state lifetimes in our mutant *Synechocystis* strains suggest that some of the variation in S_2 lifetimes in non-DCMU treated samples is due to variations in the redox state of the PQ pool rather than structural alterations of the Q_B site.

REFERENCES

Bockholt, R., Masepohl, B., & Pistorius, E. K. (1991) *FEBS Lett. 294,* 59−63.

Boerner, R. J., Nguyen, A. P., Barry, B. A., & Debus, R. J. (1992) *Biochemistry 31,* 6660−6672.

Bricker, T. M. (1992) *Biochemistry 31,* 4623−4628.

Bricker, T. M., Odom, W. R., & Queirolo, C. B. (1988) *FEBS Lett. 231,* 111−117.

Burnap, R., & Sherman, L. A. (1991) *Biochemistry 30,* 440−446.

Burnap, R., Koike, H., Sotiropoulou, G., Sherman, L. A., & Inoue, Y. (1989) *Photosynth. Res. 22,* 123−130.

Burnap, R. L., Shen, J. R., Jursinic, P. A., Inoue, Y., & Sherman, L. A. (1992a) *Biochemistry 31,* 7404−7410.

Burnap, R., Shen, J. R., Jursinic, P. A., Qian, M., Inoue, Y., & Sherman, L. A. (1992b) in *Current Research in Photosynthesis* (Murata, N., Ed.) Vol. II, pp 179−182, Kluwer Academic Publishers, Dordrecht.

Carpenter, S. D., Ohad, I., & Vermaas, W. F. J. (1993) *Biochim. Biophys. Acta 1144,* 204−212.

Chu, H.-A., Nguyen, A. P., & Debus, R. A. (1994) *Biochemistry 33,* 6137−6149.

DeVault, D., & Govindjee (1990) *Photosynth. Res. 24,* 175−181.

Eaton-Rye, J. J., & Murata, N. (1989) *Biochim. Biophys. Acta 977,* 219−226.

Enami, I., Kaneko, M., Kitamura, N., Koike, H., Sonoike, K., Inoue, I., & Katoh, S. (1991) *Biochim. Biophys. Acta 1060,* 224−232.

Frankel, L. K., & Bricker, T. M. (1992) *Biochemistry 31,* 11059−11064.

Guikema, J. A., & Sherman, L. A. (1984) *Plant Physiol. 74,* 90−95.

Ikeuchi, M., Koike, H., & Inoue, Y. (1988) *Biochim. Biophys. Acta 932,* 160−169.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82,* 488−492.

Kuwabara, T., Miyao, M., Murata, T., & Murata, N. (1985) *Biochim. Biophys. Acta 977,* 315−321.

Mayes, S. R., Cook, K. M., Self, S. J., Zhang, Z., & Barber, J. (1991) *Biochim. Biophys. Acta 1060,* 1−12.

McClary, J. A., Witney, F., & Geisselsoder, J. (1991) *BioTechniques 7,* 282−289.

Miyao, M., & Murata, N. (1989) *Biochim. Biophys. Acta 806,* 283−289.

Nixon, P., & Diner, B. (1992) *Biochemistry 31,* 942−948.

Nixon, P. J., Trost, J. T., & Diner, B. A. (1992) *Biochemistry 31,* 10859−10871.

Odom, W., & Bricker, T. M. (1992) *Biochemistry 31,* 5616−5620.

Ono, T. A., & Inoue, Y. (1984) *FEBS Lett. 166,* 381−384.

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.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular cloning: a laboratory manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Seibert, M., Tamura, N., & Inoue, Y. (1989) *Biochim. Biophys. Acta 974,* 185−191.

Seidler, A., Roll, K., & Michel, H. (1992) in *Current Research in Photosynthesis* (Murata, N. Ed.) Vol. II pp 409−412, Kluwer Academic Publishers, Dordrecht.

Shen, J.-R., & Inoue, Y. (1991) *Plant and Cell Physiol. 32,* 453−457.

Shen, J.-R., & Inoue, Y. (1993) *Biochemistry 32,* 1825−1832.

Stewart, C. A., Ljungberg, U., Akerlund, H.-A., & Andersson, B. (1985) *Biochim. Biophys. Acta 808,* 353−362.

Tanaka, S., Kawata, Y., Wada, K., & Hamaguchi, K. (1989) *Biochemistry 28,* 7188−7193.

Vass, I., Cook, K. M., Deak, Z., Mayes, S. M., & Barber, J. (1992) *Biochim. Biophys. Acta 1102,* 195−201.

Williams, J. G. K. (1988) *Methods Enzymol. 167,* 766−778.

Xu, Q., & Bricker, T. M. (1992) *J. Biol. Chem. 267,* 25816−25821.