Photoassembly of the Photosystem II $(Mn)_4$ Cluster in Site-Directed Mutants Impaired in the Binding of the Manganese-Stabilizing Protein†

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ABSTRACT: Photoactivation is the light-dependent ligation of Mn^{2+} into the H_2O oxidation complex of photosystem II (PSII) and culminates in the formation of an enzymatically active complex containing Ca^{2+} and four $Mn^{\geq 3+}$. Previous kinetic analysis demonstrated that the genetic removal of the extrinsic manganese-stabilizing protein (MSP) increases the quantum yield of photoactivation 4-fold relative to that of the wild type, consistent with the hypothesis that MSP hinders access of Mn^{2+} to a site of photoligation [Burnap, R. L., et al. (1996) *Biochemistry 35*, 874−882]. In this report, several *Synechocystis* sp. PCC6803 mutants with defined amino acid substitutions in the N-terminal region of MSP or the e-loop of intrinsic PSII protein CP47 [Putnam-Evans, C., et al. (1996) *Biochemistry 35*, 4046−4053] were characterized in terms of the binding of MSP to the intrinsic portion of the PSII complex and in terms of photoactivation kinetics. The charge-pair switch mutation, Arg384Arg385 → Glu384Glu385 in the lumenal e-loop of CP47 (CP47 RR384385EE), exhibited the most severe impairment of MSP binding, whereas the Arg384Arg385 → Gly384Gly385 (CP47 RR384385GG) mutation caused a more moderate impairment in binding. Single-substitution mutations at the highly conserved Asp9 or Asp10 positions in the amino-terminal region of MSP also resulted in a reduced binding affinity, but not as severe as that in CP47 RR384385EE. The relative quantum yield of photoactivation of hydroxylamine-extracted mutant PSII was generally found to correlate with the degree of MSP binding impairment, with the CP47 RR384385 mutants exhibiting the highest quantum yields. A two-locus, double-mutant construct involving deletion of MSP in the CP47 RR384385EE background was found to be only slightly more impaired in H_2O oxidation activity than either of the corresponding single-locus mutant derivatives, indicating that mutations at these genetically separate loci encode physically interacting products affecting the same reaction parameter during H_2O oxidation. Taken together, the results reinforce the concept that MSP interacts with the e-loop of CP47 at Arg384Arg385 and that disruption of this interaction causes significant alterations of the site of H_2O oxidation in terms of assembly and enzymatic activity of the Mn cluster.

Photosynthetic H_2O oxidation is catalyzed by the membrane-bound photosystem II (PSII) complex. The catalytic site of H_2O oxidation contains a cluster of four Mn atoms involved in the redox chemistry of the H_2O oxidation reaction [for reviews see Debus (1992), Diner and Babcock (1996), and Nugent (1996)]. The active site $(Mn)_4$ appears to be situated near the lumenal surface of the PSII complex in a region formed by intrinsic reaction center proteins and extrinsic proteins. Mutational studies indicate that the D1 protein contributes amino acid ligands to the $(Mn)_4$ (Boerner et al., 1992; Chu et al., 1995a,b; Nixon & Diner, 1992; Nixon et al., 1992). However, in addition to the D1 protein, at least four intrinsic proteins (D2, CP47, CP43, and cytochrome b_{559}) are indispensable for O_2 evolution, and none of these have been experimentally excluded from involvement in the ligation of the $(Mn)_4$ cluster.

Three water-soluble proteins are bound to the intrinsic portion of the PSII complex near the lumenal surface of the thylakoid and in close association with the active site $(Mn)_4$ [for review see Seidler (1996a)]. The proteins variously affect the binding of inorganic cofactors associated with the H_2O oxidation complex. Of these, only the manganese-stabilizing protein (MSP,[1] 33 kDa extrinsic protein) is common to all PSII-containing species. MSP is necessary for maximal rates of O_2 evolution and stabilizes active site Mn (Burnap & Sherman, 1991; Kuwabara et al., 1985; Ono & Inoue, 1984). The stoichiometry of MSP remains controversial with the existence of estimates of one per reaction center (Enami et al., 1991) and, recently, two per reaction center (Betts et al., 1997; Leuschner & Bricker,

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[1] Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, which inhibits electron transport between Q_A and Q_B; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; HA, hydroxylamine (NH_2OH); e-loop, lumenally exposed interhelical loop situated between transmembrane helices 5 and 6 of CP47; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MSP, manganese-stabilizing protein (extrinsic 33 kDa PSII protein); *psbB*, gene encoding CP47; *psbO*, gene encoding the manganese-stabilizing protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; VO_2, velocity of oxygen evolution; Y_Z, redox active tyrosine of the D1 protein acting as a secondary electron donor of the reaction center.

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1996; Xu & Bricker, 1992). Two other extrinsic proteins of 23 and 17 kDa are present in eukaryotic PSII, but are absent in cyanobacteria. Instead, cyanobacterial PSII contains two additional extrinsic proteins, cytochrome c_{550} (C550) and a 12 kDa protein (Shen & Inoue, 1993), both of which are absent in higher plant PSII. The 23 and 17 kDa eukaryotic proteins have been shown to modulate the exchange of Ca^{2+} and Cl^-, respectively, with the H_2O-splitting domain (Adelroth et al., 1995; Ghanotakis et al., 1984; Miyao & Murata, 1985). The role of the cyanobacterial C550 and 12 kDa proteins is less clear, but these proteins do not appear to have functions that closely parallel those of the 23 and 17 kDa eukaryotic proteins. Furthermore, considerable controversy surrounds the function of C550, as this protein is also postulated to function independently of PSII (Kang et al., 1994; Krogmann, 1991).

In addition to stabilizing and modulating the activity of the $(Mn)_4$ cluster, the extrinsic polypeptides influence the kinetics of its assembly (Burnap et al., 1996; Engels et al., 1994). Assembly of Mn^{2+} into the active site of the H_2O oxidation complex is a light-dependent process termed photoactivation (Cheniae & Martin, 1971a,b). During photoactivation, the valency of Mn atoms increases from Mn^{2+} to $Mn^{\geq 3+}$ as the metal atoms become coordinated within the ligation environment of the active site. The oxidation of Mn^{2+} during assembly probably occurs via the same pathway involved in the photooxidation of the fully assembled H_2O oxidation complex (i.e. via Y_z) during the catalytic cycle of H_2O oxidation (Miller & Brudvig, 1990). However, this assembly process occurs with a low quantum yield (Cheniae & Martin, 1971b; Tamura & Cheniae, 1987) (typically much less than 0.01 *in vivo*), in contrast with the relatively high quantum yields associated with charge separation and coupling to H_2O oxidation and plastoquinone reduction (typically >0.85). Genetic removal of MSP dramatically increases the quantum yield of photoactivation *in vivo* compared to that of the wild type, despite the fact that the deletion of this extrinsic polypeptide impairs the catalytic properties and stability of the assembled complex (Burnap et al., 1996). A simple interpretation of the increased quantum yield in the absence of MSP is that protein presents a diffusional barrier for Mn^{2+} atoms to the site of photooxidation. This raises the possibility that mutations affecting the binding of MSP to the reaction center complex may also alter the kinetics of the photoassembly process.

The binding of MSP to the intrinsic portion of the PSII reaction center is thought to be mediated, in part, through a direct interaction between MSP and the large (~190 amino acids) lumenal domain of CP47 situated between predicted transmembrane helices 5 and 6 (CP47 is predicted to have six transmembrane helices). Evidence for an interaction between MSP and the e-loop of CP47 comes from previous cross-linking, chemical modification, immunological, and proteolysis studies (Frankel & Bricker, 1992, 1995; Hayashi et al., 1993; Odom & Bricker, 1992; Seidler, 1996b). The amino terminus of MSP has been shown to be critical for the binding of MSP to the reaction center since proteolytic removal of the 16−18 N-terminal residues abolishes the ability of MSP to rebind (Eaton-Rye & Murata, 1989). [For reviews containing analyses of amino acid sequences of CP47 and MSP, see Bricker (1990) and Seidler (1996a), respectively.] Consistent with this finding, the CP47 e-loop (region from E364 to D440) was found to cross-link with the amino-terminal portion of MSP between E1 and K76, using the "zero-length" cross-linking reagent 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) (Odom & Bricker, 1992). Since EDC cross-links amino groups to amino groups that are within van der Waals contact, these results also suggest that the postulated binding interaction between the N terminus of MSP and the CP47 e-loop involves a charge-pair interaction between carboxyl and amino groups at the binding interface between these portions of the two proteins. The view that part of the binding site for MSP is provided by the CP47 large lumenal loop has been further strengthened by directed mutagenesis experiments modifying CP47 in that region (Haag et al., 1993; Putnam-Evans & Bricker, 1992, 1994). The work by Gleiter et al. (1994, 1995) establishes that certain short deletion mutations in the e-loop of CP47 not only affect the binding of MSP to the reaction center but also affect the dark stability of the $(Mn)_4$ clusters in the mutant PSII centers. Recent mutational analyses of the conserved charged residues of the CP47 e-loop indicate that the pair of arginine residues at positions 384 and 385 (RR384385) are most critical in the functioning of the H_2O oxidation complex, which is consistent with the hypothesis that these residues engage in the postulated ionic interaction with MSP (Putnam-Evans et al., 1996).

In this study, site-directed mutations of the RR384385 pair and a pair of conserved aspartic acid residues in the N terminus of MSP were investigated to assess possible changes in the binding affinity of MSP and whether the hypothesized changes in binding affinity are correlated with alterations in the kinetics of dark stability and photoactivation.

METHODS

The naturally transformable, glucose-utilizing strain of *Synechocystis* sp. PCC6803 (Williams, 1988) was used in the construction of the strains used in this study. The wild-type and mutant cyanobacterial experimental cultures were grown on a rotary shaker in BG-11 media supplemented with 5 mM glucose at 32 °C. Genetic manipulation of *Synechocystis* and *Escherichia coli* strains followed procedures described in Williams (1988) and Sambrook (1989), respectively. The construction of the strain designated ΔpsbO, which lacks the entire *psbO* coding sequence, has been described (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 construction and characterization of the MSP D9K mutant using oligonucleotide mutagenesis has been described in Burnap et al. (1994). A similar approach was applied to construct the set of mutations at the D10 position. Construction and characterization of the CP47 RR384385GG and CP47 RR384385EE strains is described in Putnam-Evans and Bricker (1992) and Putnam-Evans et al. (1996). Deletion of the *psbO* gene in the CP47 RR384385EE strain was accomplished by transformation with a mutagenic plasmid similar to the previous construct pRB425 (Burnap & Sherman, 1991), except that a gene cassette encoding gentamycin resistance was used to replace the *psbO* locus rather than the spectinomycin resistance cassette originally employed.

MSP binding assays were modeled on the assay described by Gleiter et al. (1994). Cells in 100 mL cultures were harvested in the late log phase of growth by pelleting at

4000*g* and resuspended to approximately 400 μg of Chl mL^{-1} in HMCS buffer (50 mM Hepes-NaOH, 5 mM $CaCl_2$, 10 mM $MgCl_2$, and 1 M sucrose) adjusted to pH 7.1 at room temperature and supplemented with 1 mM PMSF, 1 mM ϵ-caproic acid, and 1 mM benzamidine to inhibit protease activity. Cells were broken by shaking with an equal volume of 0.1 mm glass beads (Sigma Co.) in 2 mL screw-cap microfuge tubes in an aluminum adapter in a Braun Homogenizer. The homogenizer operated at top speed for 10 min with continuous agitation with the sample chamber maintained at approximately 0 °C by a stream of CO_2 from a siphon tank. Glass beads, cell debris, and unbroken cells were removed by centrifugation of the samples for 5 min at 3000 rpm in a microfuge, and the low-speed supernatants were transferred to fresh tubes. Cell lysates were adjusted to 300 μg mL^{-1}, treated with β-dodecyl maltoside at a concentration of 0.04% w/v, and (200 μL) centrifuged for 40 min at 60000*g* in a bench-top ultracentrifuge. The detergent is added to minimize entrapment of unbound MSP in membrane vesicles. Clear blue supernatants were found to contain no Chl or D1 protein, as assayed spectrophotometrically and immunologically, respectively. Treatment with higher concentrations of β-dodecyl maltoside (>0.06% w/v) resulted in some liberation of Chl and D1 to the supernatant. Supernatant from the upper three-fourths of the sample was carefully withdrawn, avoiding contamination with loosely packed membrane fragments at the pellet surface, and then used for analysis representing the supernatant fraction. The pellet was then washed with fresh buffer and then resuspended to a final volume equal to that of the initial pre-ultracentrifugal lysate (200 μL). SDS−PAGE and immunoblotting were carried out as in Burnap and Sherman (1991).

For steady-state O_2 evolution rate determinations, cells were resuspended to a chlorophyll concentration of 200 μg mL^{-1} in HN buffer (10 mM Hepes and 30 mM NaCl at pH 7.1) and maintained in dim light prior to activity measurements. Due to the tendency of oxygen evolution activity mutant cells to deactivate in the dark, this re-equilibration period was essential to establish maximal PSII activity. Maximal rates of O_2 evolution (VO_2) were determined polarographically at 30° C using a Clark-type electrode. Samples were resuspended in HN buffer supplemented with 0.6 mM DCBQ and 1 mM potassium ferricyanide, and oxygen evolution was measured in response to saturating red (>620 nm) illumination.

Hydroxylamine (HA) extraction of PSII Mn was performed according to Cheniae and Martin (1972), as in Burnap et al. (1996). Late log phase cells were harvested and resuspended in HN (10 mM Hepes and 30 mM NaCl at pH 7.0) buffer to a concentration of 200 μg of Chl mL^{-1}. Three milliliters of the resuspended cells was incubated with 2 mM HA added from a freshly prepared and neutralized 0.2 M HA stock solution. This and all subsequent steps prior to photoactivation were performed in complete darkness. After a 10 min incubation with gentle rotary shaking, the HA-treated cells were diluted with 10 mL of HN buffer and pelleted at 25 °C and 12000*g* for 6 min. The cells were resuspended in 10 mL of HNMC (10 mM Hepes, 30 mM NaCl, 1 mM $CaCl_2$, and 50 μM $MnCl_2$ at pH 7.0) buffer with gentle agitation for about 10 min. This step was repeated three times, and on the last wash, the cells were resuspended in 3 mL of HNMC buffer (200 μg of Chl mL^{-1}). Inclusion of Mn and Ca in the wash buffers was found to improve the reproducibility of the results, as noted previously (Burnap et al., 1996). All samples were kept on a shaker at 150 rpm at room temperature throughout the duration of the photoactivation experiment.

Photoactivation of HA-extracted cells was conducted at room temperature using a centrifugal bare platinum electrode using a procedure similar to that of Gleiter et al. (1995) that was used to follow the photoactivation of dark-deactivated samples. Our previous assays of photoactivation employed the use of a Clark-type electrode to monitor the light-dependent re-acquisition of O_2 evolution activity in hydroxylamine-extracted samples. Use of the bare platinum electrode to monitor the reacquisition of O_2-evolving capacity under flashing light was found to provide similar kinetic results [cf. Burnap et al. (1996)]. HA-treated cells in HN buffer were centrifuged to the surface of the electrode, and O_2 yields were measured in an apparatus as described previously (Burnap et al., 1996). Xenon flash illumination (5 μs fwhm) was determined to be saturating by following O_2 signal amplitudes in control samples as a function of flash intensity using neutral density filters. Cells remained at the electrode surface throughout the course of the experiments as shown during control experiments showing virtually no changes in the amplitudes and shape of the ampermetric signal. In contrast, samples that are intentionally disturbed (to promote partial resuspension of deposited cells) exhibit marked changes in the shape of the signal, and the amplitude of the signal from disturbed samples is decreased relative to that of the sample prior to disturbance. Additionally, little change in the amplitude of the signals of fully active untreated cells could be observed in control experiments simulating the flash illumination and intermittent polarization regimes experienced by the HA-extracted samples during the photoactivation experiments. Measurement of the development of O_2 evolution activity as a function of flash number was performed as follows. Samples on the electrode were given a train of filtered yellow (Wratten #9) xenon lamp flashes at 4 Hz. This frequency was found to give near optimal yields for all samples used in the present experiments. Immediately after the train of photoactivating flashes, the electrode was polarized, and after 10 s, a train of measuring flashes was given at 4 Hz for 5 s with the O_2 yields being recorded. The amplitudes of the O_2 yields during the last four flashes of the measuring flash train were averaged and compared with the amplitudes of the last four O_2 yields under a similar train of measuring flashes in the same sample in its maximally photoactivated state (following approximately 1000 flashes for samples exhibiting high quantum yields of photoactivation or 4000 or more flashes for samples exhibiting low quantum yields).

RESULTS

MSP Binding. Previous biochemical studies have indicated the existence of charge-pair interactions between the e-loop of CP47 and the N-terminal region of MSP (Odom & Bricker, 1992). Figure 1 shows an immunoblot experiment monitoring the fraction of MSP found in the supernatant or retained by thylakoids following high-speed centrifugation (Gleiter et al., 1994). Each mutation causes a weakened binding of MSP to the thylakoid membrane as shown by increases in the amount of MSP partitioning into the supernatant (Figure 1, lanes designated S) at the expense of

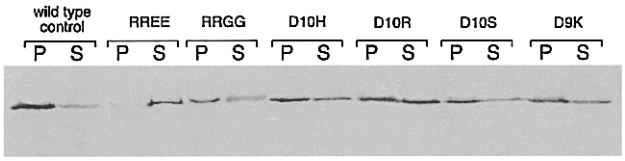

FIGURE 1: Immunoblot analysis of MSP in membrane-containing pellets and postcentrifugal supernatants in lanes designated P and S, respectively. Samples analyzed: wild-type control, wild-type *Synechocystis* sp. PCC6803; RREE, CP47 RR384385EE mutant; RRGG, CP47 RR384385GG mutant; D10H, MSP D10H; D10R, MSP D10R; D10S, MSP D10S; and D9K, MSP D9K.

Table 1

strain	$VO_{2,max}$[a]	MSP binding[b]	half-time of dark deactivation of $VO_{2,max}$ (h)[c]
wild type	100	strong	44
ΔpsbO	37	n/a	0.4
CP47 RR384385EE	44	weak	9
CP47 RR384385GG	65	moderate	22
CP47 RR384385EE/-MSP	34	n/a	0.4
MSP D9K	81	moderate	47
MSP D10H	76	moderate	48
MSP D10N	72	moderate	44
MSP D10R	78	moderate	44
MSP D10S	83	moderate	44

[a] Maximal rates of O_2 evolution ($VO_{2,max}$) expressed as a percentage of the rate observed for the wild type. The average wild-type rate (100%) was determined to be 511 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} under continuous saturating illumination and was measured at 30 °C with the addition of 600 μM DCBQ and 1 mM $K_3Fe(CN)_6$ and with samples at a concentration of 7.5 μg of chlorophyll mL^{-1}. [b] As assayed immunologically according to Figure 1. [c] $VO_{2,max}$ was measured in aliquots withdrawn a various times from samples maintained in the dark at room temperature on a rotary shaker at a concentration of 200 μg of Chl mL^{-1}.

the amount found in the pellet (Figure 1, lanes designated P). The most severe impact on MSP binding results from the double-amino acid substitution of the CP47 e-loop arginine pair RR384385 with oppositely charged glutamic acids. More moderate impacts are observed as a result of the substitution of these e-loop arginines with uncharged glycines and as a result of single-amino acid substitutions at the highly conserved aspartate 9 and aspartate 10 positions of the N terminus of the mature MSP. We concluded that each of the mutations in the N terminus and the CP47 e-loop, to varying degrees, causes decreased binding affinity of MSP for its binding site. It should be noted that a number of other site-directed mutations, also at highly conserved charged residues in MSP, do not appear to affect the binding affinity of MSP using this assay (M. Qian and R. L. Burnap, unpublished observations).

O_2 Evolution and Dark Losses of Activity. Table 1 documents maximal rates of O_2 evolution ($VO_{2,max}$) in the wild-type and mutant strains. Similar to previous observations on the MSP D9K mutant, amino acid substitution mutations at positions D10 do not severely impact $VO_{2,max}$ (Burnap et al., 1994; Seidler et al., 1996). In agreement with previous results, the MSP-less mutant (strain ΔpsbO) and the CP47 RR384385EE mutant exhibit maximal rates of O_2 evolution that are 37 and 44% of the wild-type rate, respectively (Burnap & Sherman, 1991; Putnam-Evans et al., 1996). Since the above binding experiments indicate that the CP47 RR384385EE mutation dramatically impacts the binding of MSP, we reasoned that the genetic removal of MSP from the CP47 RR384385EE strain would not further reduce the maximal rate of O_2 evolution by the full 63% expected if the mutations of the CP47 e-loop and MSP were independent. The deletion of MSP in the CP47 RR384385EE (strain CP47 RR384385EE/-MSP) background produced an only marginal further decrease in the maximal rate of O_2 evolution, to about 34% of the wild-type level, rather than the $(0.37 \times 0.44) \times 100$ (=16%) predicted if the impacts of the two mutations were independent. Therefore, we conclude that the effects of the charge switches at RR384385 on the e-loop of CP47 and the genetic removal of MSP are not independent. Thus, these mutations appear to negatively impact the function of the H_2O oxidation complex by affecting the same parameter(s) governing the reaction mechanism.

Table 1 also documents the dark stability characteristics of the H_2O oxidation activity in each of the mutants examined. Previous studies demonstrated that the absence of MSP (ΔpsbO strain) leads to rapid ($t_{1/2}$ = 10−20 min) losses of H_2O oxidation activity, which is assumed to correlate with loss of active site Mn in analogy with *in vitro* systems. Consistent with this, very rapid deactivation was also observed for CP47 RR384385EE/-MSP (Table 1). Although very rapid deactivation was not observed for the other mutant strains, the RR384385EE and RR384385GG mutants exhibited intermediate dark stabilities, with half-times of approximately 9 and 22 h, respectively. In contrast, the D9 and D10 MSP mutants exhibited dark stability characteristics similar to those of the wild type, showing little loss in activity even after 24 h in the dark. From these results, we suggest that MSP remains associated with the H_2O oxidation complex despite the weakened binding affinity.

Photoassembly of $(Mn)_4$ Clusters. Photactivation was analyzed in cells extracted with hydroxylamine (HA) to remove PSII active site Mn (Burnap et al., 1996; Cheniae & Martin, 1971a, 1972). This extraction was performed under conditions promoting the retention of Mn^{2+}, Ca^{2+}, and Cl^- within the cells to ensure the presence of these necessary cofactors for the subsequent photoassembly of $(Mn)_4$ clusters, which in this case was driven by saturating flash illumination. The experiments were designed to determine whether the site-directed mutations described here affect the kinetics of photoassembly of the $(Mn)_4$ cluster. The flash number dependence of photoactivation provides a measure of the relative quantum yield of photoactivation as defined as the per flash increase in the fraction of PSII centers capable of evolving O_2. Consistent with previous results (Burnap et al., 1996), the genetic removal of MSP (ΔpsbO mutant strain) increases the relative quantum yield of photoactivation (Figure 2). This difference in kinetics is not due to changes in local concentrations of the Mn^{2+} or Ca^{2+} ions, since the similar differences in relative quantum yield between the ΔpsbO and the wild type are observed in samples that have been HA-extracted in the presence of EGTA and the divalent cation ionophore A23187; this treatment renders photoactivation dependent upon addition of exogenous Mn^{2+} and Ca^{2+}, and photoactivation using optimal concentrations of these cations also occurs with an approximately 4-fold higher relative quantum yield in the ΔpsbO strain than in the wild type (M. Qian and R. L. Burnap, unpublished observations).

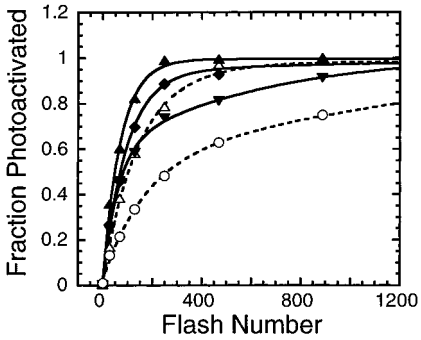

FIGURE 2: Photoactivation as a function of flash number in HA-treated cells with mutations in the e-loop of CP47: CP47 RR384385GG (▼), CP47 RR384385EE (◆), CP47 RR384385EE/-MSP (▲), ΔpsbO (△), and wild type (○). The photoactivating light consisted of a sequence of saturating, single-turnover xenon actinic flashes given at a fixed frequency of 4 Hz. Cells were centrifugally deposited on the electrode surface and subjected to a photoactivating flash sequence given without electrode polarization. Data points represent the normalized amplitudes of the O_2 signal obtained as the O_2 signal amplitude after the photoactivating sequence divided by the O_2 signal amplitude in the fully photoactivated sample. Data points represent the averages of at least three experiments, and the standard deviations at any point do not exceed 0.06 of maximal (1.0).

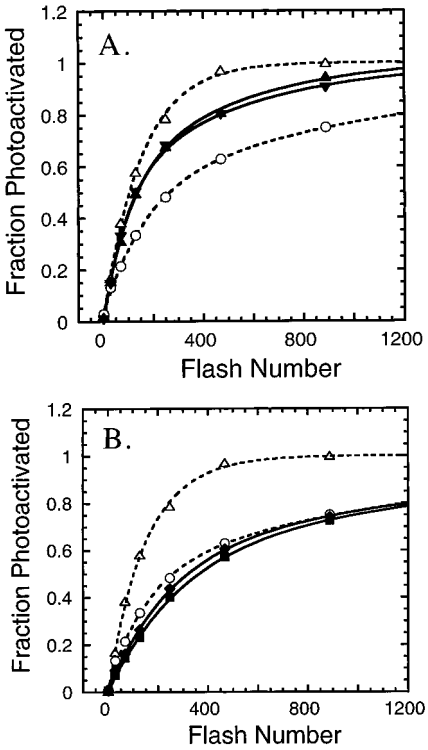

FIGURE 3: Photoactivation as a function of flash number in HA-treated cells with mutations in the N-terminal region of MSP. Photoactivation was performed and presented as in Figure 2: (A) MSP D10H (▼), MSP D10S (▲), D9K ΔpsbO (△), and wild type (○); and (B) MSP D9K (◆), MSP D10R (■), ΔpsbO (△), and wild type (○). Data points represent the averages of at least three experiments, and the standard deviations at any point do not exceed 0.11 of maximal (1.0).

As shown in Figure 2., relative quantum yields exceeding even that of the ΔpsbO strain were observed for each of the CP47 mutants involving substitutions of the basic charge pair RR384385. The CP47 RR384385EE/-MSP strain exhibited the highest relative quantum yield of all strains examined.

As shown in Figure 3A, the quantum yield of photoactivation of the D10H, D10N, and D10S mutants was higher

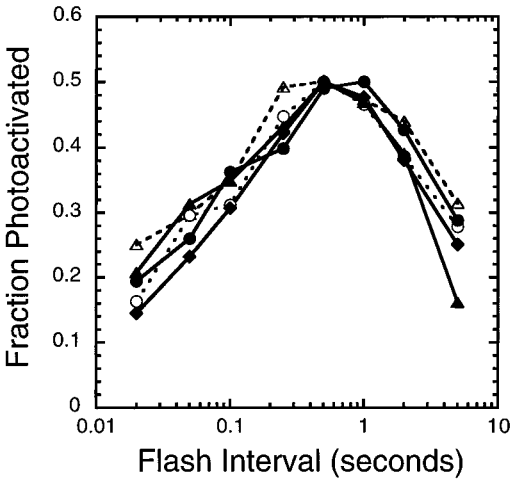

FIGURE 4: Photoactivation as a function of flash interval in HA-treated cells with mutations in the e-loop of CP47: CP47 RR384385GG (◆), CP47 RR384385EE (●), CP47 RR384385EE/-MSP (▲), ΔpsbO (△), and wild type (○). The samples were given fixed numbers of flashes at different flash intervals ranging from 50 ms to 4 s. The number of flashes given to each sample was set to produce approximately 50% maximal photoactivation at 4 Hz for that particular sample as determined in the flash number dependence experiments (Figure 2). After the fixed number of flashes was given at the indicated flash interval, the electrode polarization was turned on and a 4 Hz measuring flash sequence was applied as described in Figure 2. The electrode polarization was switched off, and additional flashes were applied at 4 Hz to promote maximal photoactivation. The data points are presented as the O_2 signal amplitudes obtained after the fixed-number, variable interval, photoactivating flash sequence divided by the O_2 signal amplitudes at maximal photoactivation of the sample. Data points represent the averages of at least three experiments, and the standard deviations at any point do not exceed 0.17 of maximal (1.0).

than that of the wild type, although not to the extent observed for ΔpsbO. On the other hand, replacement of the negatively charged aspartate with oppositely charged residues in mutant strains D9K and the D10R (Figure 3B) did not produce an increase, but instead decreased the quantum yield despite the fact that these charge-switch mutations decrease the binding affinity to an even greater extent than other substitutions at these positions (Figure 1).

To test whether the kinetics of the rate-limiting steps and the lifetimes of unstable intermediate photoproducts of the multiquantum photoactivation process have been altered by the mutations, the flash interval dependence of photoactivation was measured. Figure 4 shows the extent of photoactivation resulting from a fixed number of flashes given at different flash intervals in the wild-type, ΔpsbO, and CP47 mutant strains. Very similar results were observed for the MSP N-terminal mutants (not shown). The wild-type and mutant strains all exhibit the characteristic bell-shaped curve, presented in normalized form, observed both *in vivo* (Burnap et al., 1996; Cheniae & Martin, 1972; Ono & Inoue, 1983) and *in vitro* (Ananyev & Dismukes, 1996a,b; Miyao & Inoue, 1991; Tamura & Cheniae, 1987; Tamura et al., 1989). The slope of the descending portion provides an estimate of the lifetime of the unstable intermediate, whereas the slope of the ascending portion of the curve is related to the rate constant of this dark step(s) (Cheniae & Martin, 1971b, 1972; Miyao & Inoue, 1991). Relatively lower yields of assembled centers are obtained for all strains when the spacing of flashes is very short (Δt < 50 ms). This phenomenon reflects the existence of a low-quantum yield process initiating a dark

step(s) that must go to completion before effective utilization of the next quantum occurs. Higher yields at very short flash intervals are obtained in most of the mutants (ΔpsbO > RR384385EE ≅ RR384385EE/-MSP > RR384385GG ≅ wild type). The higher yields at the shortest flash intervals are largely consistent with the higher relative quantum yields seen in Figures 2 and 3 and suggest that centers are more efficiently initiated or stabilized by some light-dependent step along the assembly pathway. However, these differences approach the range of experimental error and thus should be interpreted cautiously. Lower yields seen in all samples at long flash intervals (Figure 4) also reflect the multiquantum requirement of photoactivation, since the product of the first quantum absorbed by the center is expected to have a greater probability of decaying if a second quantum arrives at a long interval after the first. Most of the mutants however do not show large departures from the assembly intermediate decay kinetics relative to the wild type, with the exception of the RR384385EE/-MSP mutant, which clearly exhibits a significantly destabilized assembly intermediate shown by the comparatively lower yields at long flash intervals.

DISCUSSION

MSP Binding. Previous biochemical analysis has indicated that the e-loop of CP47 provides critical determinants for the binding of MSP to the PSII reaction center complex [reviewed in Bricker (1990), Debus (1992), and Seidler (1996a)]. On the other hand, the N-terminal portion of MSP is critical for the binding of the protein to the reaction center and may be cross-linked to CP47. In this work, we have analyzed genetically engineered mutants having amino acid substitutions which hypothetically alter the binding interface between MSP and the e-loop of CP47. The results of the *in vitro* binding assays, as illustrated in Figure 1, demonstrate that the basic pair of arginyl residues in the CP47 e-loop at positions 384 and 385 (Arg384Arg385) is crucial for the stable binding of MSP to the PSII reaction center. Previous work had shown that mutation of this basic charge pair impairs the function of the H_2O oxidation complex, resulting in a lowered $VO_{2,max}$, a retarded S_3-S_0 transition, and a greater susceptibility to photoinhibition (Putnam-Evans & Bricker, 1992; Putnam-Evans et al., 1996). In fact, among all of the single mutations of potentially charged residues within the e-loop, only mutations at Arg384 and Arg385 resulted in severe impairment of H_2O oxidation activity (Putnam-Evans et al., 1996). In this study, mutation of these residues resulted in severe decreases in the binding of MSP to the reaction center. Using an alternative mutagenesis strategy involving the production of short deletion (three to eight amino acids) mutations, Gleiter et al. (1994) discovered that removal of amino acids Ala373−Asp380 (strain designated Δ8) resulted in weakened binding of MSP. Taken together, these studies provide strong genetic evidence that this region of the e-loop is critical for MSP binding and support the hypothesis that the primary interaction with MSP involves the Arg384Arg385 residues. These conclusions are further strengthened by the observation that deletion of MSP in the CP47 RR384385EE genetic background only causes a relatively small further decrease in the $VO_{2,max}$ relative to that of the parental CP47 RR384385EE strain, indicating that the deletion of MSP and alteration of Arg384Arg385 are not independent mutations. The lack of independence suggests that MSP and Arg384Arg385 physically interact and mutation of either locus produces similar physical effects. Consistent with this, a number of impacts of the Arg384Arg385 mutations (i.e. lowered $VO_{2,max}$, a retarded S_3-S_0 transition, and a greater susceptibility to photoinhibition) resemble those observed for ΔpsbO (Putnam-Evans et al., 1996).

The weakening of MSP binding due to the CP47 RR384385EE and CP47 RR384385GG mutations is consistent with their involvement in a direct interaction with MSP, although we cannot presently exclude the possibility that observed effects of the mutation are due to an allosteric effect upon the conformation of the proteins involved. Several results bear on this issue. The use of the "zero-length" cross-linking reagent 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) has led to the conclusion that carboxylic and primary amino groups are responsible, in part, for binding the domain Glu364−Asp440 in the CP47 e-loop to the domain Glu1−Lys76 of MSP (Odom & Bricker, 1992; Seidler, 1996b). However, EDC identifies contacts between carboxyl groups and primary amino groups (lysine, N-terminal amino group) and therefore will not identify interactions involving arginyl residues, and thus, the EDC cross-link(s) identified by Odom and Bricker between the N terminus of MSP and the CP47 e-loop does not directly involve the Arg384Arg385 residues investigated here. Since it appears likely that the Arg384Arg385 residues are involved in a direct interaction with MSP, as discussed below, it is likely that multiple interactions involving charged residues are responsible for binding MSP to the CP47 e-loop. Recent experiments by Seidler (1996b) using EDC have suggested that primary amino groups involved in the charge-pair interaction between CP47 and MSP are all located on CP47 and/or an unidentified small intrinsic protein, whereas the carboxylate groups participating in the binding interactions are situated on MSP. Taken together with the results of Odum and Bricker (1992), this suggests that carboxylate residues within the first 77 amino acids of MSP are in van der Waals contact with amino groups of lysyl side chains within the Glu364−Asp440 domain of the e-loop. However, these conclusions are in direct conflict with conclusions recently reached by Miura et al. (1997), who utilized different cross-linking reagents. The results of Miura et al. indicate that positively charged groups of MSP and negatively charged groups of the intrinsic polypeptides are critical for the MSP−reaction center binding interaction. The reason for this discrepancy remains to be established, yet we note that Figure 1 data of Miura et al. (1997) do show that some carboxylate residues on MSP are blocked by the binding of MSP to the reaction center, consistent with the conclusions of Odum and Bricker (1992) and Seidler (1996b).

In contrast to the severe effects on the binding of MSP and $VO_{2,max}$ resulting from the CP47 RR384385EE mutation, we find here that the mutations of aspartic acid residues D9 and D10 within the N terminus of MSP only produced moderate reductions both in $VO_{2,max}$ and in the affinity of MSP binding to the reaction center. Furthermore, these rather moderate effects were observed even when the residues were replaced with oppositely charged groups as in the D9K and D10R strains. The reduced MSP binding affinities of the different mutations at D9 and D10 were roughly equivalent to one another and comparable to that of the CP47 RR384385GG mutant. The reduced binding affinity presumably explains the lability of H_2O oxidation activity during

the isolation of thylakoid membranes from these strains (Burnap et al., 1994; M. Qian and R. L. Burnap, unpublished data). In contrast to our observations of reduced binding affinity in the *Synechocystis* MSP N-terminal mutants, Seidler et al. (1996) did not observe a difference between the D9 mutant and wild-type MSP utilizing heterologously expressed MSP reconstituted into spinach PSII *in vitro*. That assay followed the restoration of $VO_{2,max}$ as a function of MSP concentration. The basis for these disparate results is not clear; however, neither the assays described here nor those of Seidler were performed under truly equilibrium binding conditions; development of such an assay should clarify the apparent discrepancy.

What are the likely interactions that have been affected by the mutagenesis procedures employed in this study? The fact that low pH can induce the dissociation of MSP is consistent with the energy of the binding interaction being dominated by electrostatic interactions (Shen & Inoue, 1991), although other interactions such as hydrophobic interactions are also likely to be involved given other results (Kuwabara et al., 1985).

In the case of the very tight binding complex between barnase and barnstar, coupling is energetically ($\Delta G = -19$ kcal mol^{-1} at 25 °C) driven by polar interactions between the largely negative surface charge of the barnstar interfacial surface and the largely positive interfacial surface of barnase (Schreiber & Fersht, 1993, 1995). Networks of directional hydrogen bonds between acidic and basic groups located at the innermost regions of contact provide the strongest interactions, whereas salt bridges located at the periphery of the interface surface are energetically less significant (Schreiber & Fersht, 1995). A given charged residue at the interface may engage several different types of specific interaction and typically with several different partners. For example, aspartate 39 of barnstar hydrogen bonds to barnase residues histidine 102, arginine 83, and arginine 87, with the interactions with the latter two residues being especially strong ($\Delta G \cong -6$ kcal mol^{-1}), and the strength of these interactions can be partly attributed to their isolation from solvent at the interior of the interface (Schreiber & Fersht, 1995). Given the key role of the Arg384Arg385 pair in MSP binding, it is tempting to speculate that these residues are involved in similar interactions. The CP47 RR384385EE double-charge switch mutant results in a profound decrease in MSP binding affinity, consistent with the proposition that the Arg384Arg385 pair engages in an intermolecular cross-link with MSP that, when mutated to negative charges, overwhelms the remainder of the energy of binding MSP to the reaction center due to electrostatic repulsion. It is clear from our results that each of the various mutations in D9 and D10 results in reductions, albeit much smaller, in MSP binding affinity. If these residues are engaged in intermolecular interactions, then they make only a marginal contribution to the overall binding affinity of MSP. While ionic interactions may have bond energies on the order of -5 to -10 kcal mol^{-1} depending on the local dielectric, the free energy of ionic bond formation, taking into account the energetic cost of desolvating ions, makes the contribution of individual ionic bonds much less, typically $\sim$1 kcal mol^{-1} (Schulz & Schirmer, 1979). Considering that the free energy of MSP binding to the reaction center has been estimated to be about -10 and -9 kcal with and without an intact $(Mn)_4$, disruption of a single ionic bond should only marginally affect the total binding free energy (Leuschner & Bricker, 1996; Miyao & Murata, 1989). However, if the mutation is a charge switch mutation, then the effect of electrostatic repulsion, not simply the loss of electrostatic attraction, must be considered. Thus, the energetic cost of repulsion plus the energetic cost of desolvation combine to destabilize intermolecular binding. In the case of the D9K and D10R charge switch mutations in the N terminus of MSP, however, radical decreases in MSP binding affinity are not observed (Figure 1). Therefore, it seems less likely that these residues are involved in intermolecular ionic bonding interactions, at least if no conformational rearrangements are allowed to accommodate the unfavorable electrostatics. In his study of site-directed mutations of MSP and their effect upon EDC cross-linking, Seidler et al. (1996) concluded that D9 is involved in an intramolecular charge-pair interaction, the disruption of which rendered the N terminus of the protein more susceptible to proteolysis. This would suggest that the weakened binding we observed in our D9K mutant is due to a change in the tertiary structure of MSP rather than to an alteration in an ionic interaction between MSP and the reaction center core. Although this is a reasonable interpretation, it is worth considering the possibility that the D9 and D10 residues of MSP may each engage in multiple interactions [i.e. their interactions have significant multipolar character; see for example Schreiber and Fersht (1995)]. According to this view, a given charged group may engage in both intra- and intermolecular interactions.

Photoassembly of $(Mn)_4$ in the Mutants. While these experiments show that site-directed mutations in the N-terminal region of MSP and in the e-loop of CP47 alter the binding affinity of MSP for the reaction center, they also show that alterations in binding affinity correlate, in most cases, with alterations in the quantum yield of photoactivation. Burnap et al. (1996) and Engels et al. (1994) previously observed that genetic removal of MSP in *Synechocystis* sp. PCC6803 caused significant changes in the kinetics of photoassembly of the PSII $(Mn)_4$. Specifically, the quantum yield of photoactivation, which is characteristically low in all species examined to date, was increased approximately 4-fold upon deletion of MSP (Burnap et al., 1996). On the other hand, the kinetics of the dark rearrangement step of photoassembly appeared to be largely unaffected by the absence of this extrinsic protein. Consistent with these earlier results, we found that strains that entirely lack MSP (ΔpsbO and CP47 RR384385EE-ΔpsbO) exhibit high quantum yields of photoactivation. The CP47 RR384385EE and CP47 RR384385GG strains also exhibited very high quantum yields of photoactivation, correlating with the very weak binding of MSP in these strains. The *Synechocystis* mutants with defined amino acid substitutions in the N-terminal region exhibited quantum yields intermediate between those of the wild type and ΔpsbO, correlating with the intermediate MSP binding affinities observed in these mutants. The D9K and D10R mutants represent interesting exceptions to the correlation between reduced binding affinity and relative higher quantum yields of photoactivation as discussed below.

What is the basis for the increased quantum yield of photoactivation in the mutants? Control experiments testing the effects of Ca^{2+} and Mn^{2+} on photoactivation in HA/EGTA/A23187-treated cells eliminate the possibility that the observed differences in relative quantum yield are due to differences in the availability of these cations (M. Qian and

R. L. Burnap, unpublished data). Instead, we have argued that absence of MSP (Burnap et al., 1996) or, in this case, the altered binding of MSP renders the active site more accessible to incoming Mn^{2+}. Increased accessibility of the active site of H_2O oxidation is shown by a variety of studies showing that the extrinsic PSII polypeptides shield the $(Mn)_4$ from attack by exogenous reductants and the carboxyl ligands to the $(Mn)_4$ from chemical modification. The increased accessibility of the active site due to the genetic perturbations studied here is hypothesized to result in a higher probability that oxidizing equivalents generated by the reaction center are utilized for the photooxidation and ligation of Mn atoms rather than becoming dissipated via charge recombination. Alternatively, the mutations may result in a more long-lived charge-separated state, (e.g. by exposing the charges to the bulk dielectric, thereby stabilizing them) which would the increase the probability of productive photooxidation of Mn^{2+} rather than nonproductive charge recombination. A third alternative for the increased quantum yield of photoactivation is that MSP binding affects protonation/deprotonation events necessary for photoassembly. The experiments of Ananyev and Dismukes (1996b) indicate that productive photoligation requires ionization of one or more protons following formation of the first unstable intermediate and prior to the formation of the second unstable intermediate. Since our earlier work indicates that the absence of MSP affects the quantum yield of photooxidation of the second unstable intermediate, it is possible that MSP alters the ionization of the critical protons. Hence, the absence of MSP may permit more facile deprotonation of the critical ionizable groups and/or shift their pK_a of the ionized/un-ionized equilibrium toward the deprotonated state.

The D9K and D10R mutants exhibited quantum yields of photoactivation even lower than that of the wild type despite the fact that the mutations reduced the binding affinity to a degree similar to that observed for mutants with high quantum yields. In their study of the effect of tetraphenylboron effects on photoactivation, Ananyev and Dismukes (1996b) concluded that the surface charge near the site of Mn^{2+} photooxidation influences the photoassembly process via electrostatic steering effects. Similarly, we speculate that D10 and D10 are near the site of Mn^{2+} photooxidation and modulate the electrostatic environment of the H_2O-oxidase in a manner that determines the quantum yield. For the same reasons, we speculate that the very high quantum yields of photoactivation of the CP47 RR384385EE and CP47 RR384385EE/-MSP mutants are due to the location of Arg384Arg385 near the site of Mn^{2+} photooxidation, thus bringing positive charge to the electrostatic environment and thereby causing repulsion of incoming Mn^{2+}. Replacement of these basic residues with acidic residues may increase the local concentration of Mn^{2+} contributing to the very high quantum yield of photoactivation observed for these mutants. If these ideas are correct, this would place MSP D9 and D10 and CP47 RR384385 all close to the site of photooxidation of incoming Mn^{2+}.

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