Site-Directed Mutagenesis of Basic Arginine Residues 305 and 342 in the CP 43 Protein of Photosystem II Affects Oxygen-Evolving Activity in *Synechocystis* 6803†

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ABSTRACT: The intrinsic chlorophyll protein CP 43, a component of photosystem II (PS II) in higher plants, green algae, and cyanobacteria, is encoded by the *psbC* gene. Oligonucleotide-directed mutagenesis was employed to introduce mutations into a segment of *psbC* that encodes the large extrinsic loop E of CP 43 in the cyanobacterium *Synechocystis* 6803. Two mutations, R305S and R342S, each produced a strain with impaired photosystem II activity. The R305S mutant strain grew photoautotrophically at rates comparable to the control strain. Immunological analyses of a number of PSII components indicated that this mutant accumulated normal quantities of PSII proteins. However, this mutant evolved oxygen to only 70% of control rates at saturating light intensities. Measurements of total variable fluorescence yield indicated that this mutant assembled approximately 70% of the PSII centers found in the control strain. The R342S mutant failed to grow photoautotrophically and exhibited no capacity for oxygen evolution. However, when grown photoheterotrophically in medium containing both glucose and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), oxygen-evolving activity was observed in the R342S mutant, but at a low level of approximately 10% of the control rate. Immunological analysis of isolated thylakoid membranes from this mutant also indicated that this strain accumulated normal amounts of PSII core proteins. Total variable fluorescence yields for the R342S mutant indicated that it assembled a severely reduced number of fully functional PSII centers. R305S and R342S mutant strains exhibited, respectively, 2.7- and 4-fold increased sensitivity to photoinactivation. The fluorescence rise times for both mutants were comparable to the control when hydroxylamine was used as electron donor. However, both strains exhibited an increase (2.5- and 8-fold, respectively, for R305S and R342S) in fluorescence rise times with water as an electron donor. These results suggest that the mutations R305S and R342S each produce a defect associated with the oxygen-evolving complex of photosystem II. These are the first site-directed mutations in CP 43 to show such an effect.

The photosystem II (PSII)[1] complex catalyzes the light-driven oxidation of water with concomitant reduction of plastoquinone to plastoquinol. This multisubunit protein−pigment complex contains both intrinsic and extrinsic protein components. The intrinsic components associated with the thylakoid membrane that are absolutely required for the formation of a PSII complex competent to evolve oxygen are CP 47 (49 kDa), CP 43 (45 kDa), D1 (34 kDa), D2 (32 kDa), and the α and β subunits of cytochrome b_{559} (9 and 4.5 kDa) (*1*, *2*). In higher plants, the presence of three extrinsic proteins of molecular masses 33, 24, and 17 kDa allows for maximal rates of oxygen evolution under physiological conditions (*3*). Cyanobacteria lack the 24 and 17 kDa proteins. The functions of these components may be provided by cytochrome c_{550} (17 kDa) and a 12 kDa component (*4*). Several ionic cofactors are also required for oxygen-evolving activity. These include four manganese, one or two calcium, and one, two, or several chloride ions. The binding sites for these cofactors remain undetermined (*5*).

The product of the *psbC* gene, CP 43, is an integral thylakoid protein and component of the proximal antennae of PSII (*6*). Hydropathy analysis predicts that CP 43 contains six transmembrane α helices (*7*). In addition to the six membrane-spanning regions, CP 43 also contains five hydrophilic loops that connect the membrane-spanning domains. One of these loops, the large extrinsic loop E, spans amino acid residues ^{293}Asn−^{425}Arg and is located between the fifth and sixth membrane-spanning helices. This large extrinsic loop is exposed to the lumenal side of the thylakoid membrane (*8*). One function of CP 43 is as an interior chlorophyll *a* light harvesting antennae for PSII. CP 43 may also play roles in the stable assembly of PS II and in the oxygen-evolving process. To date, all oxygen-evolving PSII preparations that have been isolated contain CP 43 (*9*). *Chlamydomonas* mutants bearing alterations in *psbC* that affect either the synthesis or stability of CP 43 are deficient in PSII activity (*10*). In these mutants, the levels of other

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[1] Abbreviations: PSII, photosystem II; kb, kilobase pairs; DCBQ, 2,6-dichloro-*p*-benzoquinone; PCR, polymerase chain reaction; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; kDa, kilodaltons; LDS, lithium dodecyl sulfate; HA, hydroxylamine.

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PSII core proteins are severely reduced. *Synechocystis* mutants lacking the *psbC* gene due to deletion mutagenesis accumulate PSII core complexes lacking CP 43 to only 10% of wild-type levels and cannot grow photoautotrophically or evolve oxygen (*11*, *12*), suggesting that CP 43 is essential for normal PSII assembly and function. These *Synechocystis* mutants lacking CP 43 do support electron transport from Z to Q_A (*12*). However, electron transport from Q_A to Q_B is impaired and this was suggested to account for the loss of oxygen-evolving activity. Interruption of the *psbC* gene by insertional mutagenesis also produced a mutant incapable of evolving oxygen but that did exhibit primary charge separation (*13*). Following nitrosoguanidine mutagenesis, Dzelzkalns and Bogorad (*14*) recovered a *Synechocystis* mutant unable to evolve oxygen or support PSII electron transport from water to either dichlorobenzoquinone (DCBQ) or methyl viologen. Further characterization of this mutant showed that it resulted from a short deletion within the *psbC* gene. Additionally, isolated thylakoid membranes from this mutant exhibited decreased levels of the reaction center protein D2.

Kuhn and Vermaas (*15*) introduced eight short deletions within the large extrinsic loop E of CP 43 in *Synechocystis*. Significantly, all resulting mutants showed complete loss of photoautotrophic growth and the ability to evolve oxygen. These mutants also contained decreased levels of the PSII reaction center proteins D1, D2, and CP 47. The large extrinsic loop of CP 43 may be associated with the manganese-stabilizing protein. This loop is shielded from tryptic attack in the presence of the manganese-stabilizing protein but not in its absence (*16*). These data infer a role for the large extrinsic loop of CP 43 in normal PSII function. The loop contains a number of conserved charged amino acid residues (*7*). It is possible that some of these provide sites of interaction of CP 43 with the manganese-stabilizing protein or other protein components or ionic cofactors. We have targeted these conserved charged residues within the loop for mutagenesis in order to determine their potential roles in CP 43 function. Here we report that alteration of the basic residues ^{305}R and ^{342}R produces mutants with impaired PSII activity associated with a defect at the oxygen-evolving site of PSII.

EXPERIMENTAL PROCEDURES

Growth Conditions. Wild-type and mutant *Synechocystis* sp. PCC 6803 were grown in liquid BG-11 medium (*17*) at 30 °C and a light intensity of 25 μmol of photons$\cdot$m$^{-2}\cdot$s^{-1}. Cultures were shaken on a rotary shaker at 200 rpm. Where appropriate, glucose was added to cultures to a final concentration of 5 mM. For fluorescence measurements, cells were grown in BG-11 containing 5 mM glucose and 10 μM DCMU. Antibiotics were added to the medium to a final concentration of 10 μg/mL. Cultures maintained on plates in BG-11 medium were supplemented with 1.5% agar, 0.3% sodium thiosulfate, 10 mM TES/KOH, pH 8.2, and 10 μM DCMU.

Site-Directed Mutagenesis. Standard procedures (*18*) were employed for restriction digests, cloning, growth, and transformation of bacterial strains and isolation of DNA fragments. Phagemid isolations were performed by using disposable anion-exchange columns (Qiagen, Inc., and Promega, Inc.).

Construction of the pTZ*psbC* phagemid used for mutagenesis was as described in ref *19*. Briefly, a 2.4 kb *Acc*I/*Acc*I fragment of *psbC* from the plasmid pD1CK (kindly provided by Dr. Wim Vermaas), encoding the C-terminal portion of CP 43, was cloned into the multiple cloning site of the phagemid pTZ18U (Bio-Rad). This fragment contained the entire large extrinsic loop coding region as well as 0.32 kb of *psbC* coding sequence to the 5′ end and 0.36 kb of 3′ flanking sequence. A 1.2 kb kanamycin resistance cartridge was inserted into the 3′ flanking region and was located 173 bp from the 3′ end of the *psbC* coding region. Transformation of wild-type *Synechocystis* with this phagemid, designated pTZ*psbC*, resulted in the kanamycin-resistant control strain, which contained an intact *psbC* gene but no site-directed mutations. Desired mutations were introduced into pTZ*psbC* by oligonucleotide-directed mutagenesis by the procedure of Kunkel et al. (*20*). Synthetic oligonucleotides were constructed to give the requisite arginine to serine changes (see Results). Mutant phagemids were sequenced (see below) to confirm the presence of the desired mutations prior to transformation into wild-type *Synechocystis*. Transformations were carried out by the procedure of Williams (*21*). Colonies of putative site-directed mutants were screened for kanamycin resistance and also streaked on kanamycin-containing plates in the presence or absence of glucose to screen for possible loss of photoautotrophic growth.

Following verification of the mutant phenotypes, R305Sr and R342Sr revertant strains were constructed by transformation of each site-directed mutant with the nonmutagenized pTZ*psbC* phagemid. This in effect "reverted" each strain to wild type by converting the site-directed mutations back to the corresponding wild-type codons. R342Sr colonies were selected for initially by the restoration of photoautotrophic growth (followed by DNA sequencing). R305Sr colonies were identified solely by sequencing of the genomic DNA.

PCR and DNA Sequencing. To verify the presence of intended mutations, genomic DNA was isolated from putative mutants from cell lysates according to the procedure of Williams (*21*) except that the cesium chloride steps were omitted. Oligonucleotides flanking the *Acc*I/*Acc*I fragment of the *psbC* gene were used to amplify this region of the genomic DNA of each mutant by the polymerase chain reaction. The thermal cycling routine consisted of the following steps performed on 52 μL reactions: 1 min of denaturation at 94 °C, 40 s of denaturation at 92 °C, 40 s of annealing at 45 °C, and 1.5 min of elongation at 75 °C, for a total of 30 cycles. PCR products were cloned into the pGEM-T vector (Promega). Plasmids with inserts were sequenced with the PRISM Ready Reaction Dyedeoxy Terminator Cycle sequencing kit (Applied Biosystems, Inc.). The sequencing reactions were analyzed on an automated DNA sequencer (Model 373A, Applied Biosystems).

Oxygen Evolution Assays. PSII activity was measured by O_2 polarography with a Hansatech oxygen electrode. Assays were performed at 25 °C on whole cells in BG-11 medium with 1 mM DCBQ as an electron acceptor. The light intensity for these experiments was 2500 μmol of photons$\cdot$m$^{-2}\cdot$s^{-1} of white light. Light intensity was measured with a spectroradiometer equipped with a quantum probe (Li-Cor, Inc.). For photoinactivation experiments, cells were incubated in BG-11 medium at a chlorophyll concentration of 10 μg/mL at 5000 μmol photons$\cdot$m$^{-2}\cdot$s^{-1} at 25 °C. At the indicated

times, aliquots were removed and assayed for oxygen-evolving activity as described above. The chlorophyll concentration in all oxygen evolution assays was 10 μg/mL. Chlorophyll content was measured as in Williams (*21*).

Fluorescence Measurements. Fluorescence yield measurements were performed on a Walz PAM 101 fluorometer as described previously (*22*, *23*). Samples (10 μg/mL chlorophyll) were incubated in the dark for 5 minutes in the presence of 1 mM potassium ferricyanide and 330 μM DCBQ. DCMU was added to a final concentration of 40 μM followed 1 minute later by the addition of hydroxylamine hydrochloride (pH 6.5) to a concentration of 20 mM. After 20 s the weak monitoring flashes were turned on followed 1 s later by continuous actinic illumination (1000 μmol of photons$\cdot m^{-2}\cdot s^{-1}$). The variable fluorescence, $F_{v(HA)}$, was measured. A second set of assays was performed as above except that the hydroxylamine was omitted in order to measure the variable fluorescence, $F_{v(H_2O)}$, with water as the electron donor. In both cases, F_{max} was measured 5 s after the onset of actinic illumination.

Electrophoresis and Immunological Analysis. Thylakoid membranes were isolated according to the method of van der Bolt and Vermaas (*24*). These membranes were subjected to electrophoresis on LDS−polyacrylamide gels, Western blotted onto nitrocellulose, and probed with antibodies to PSII core proteins essentially as described by Bricker et al. (*25*), except that the secondary antibodies were alkaline phosphatase conjugates. The protein bands were visualized by incubation with alkaline phosphatase-conjugated secondary antibodies and color development with bromochloroindoyl phosphate and nitroblue tetrazolium as described by Pratt et al. (*26*). The anti-CP 43 antibody was a gift from Dr. N.-H. Chua and was raised against the purified polypeptide from *Chlamydomonas* (*27*). The anti-CP 47 antibody was raised against purified spinach CP 47 (C. Leuschner, L. Sherman, and T. M. Bricker, unpublished results). The anti-D1 antibody was supplied by Dr. A. Trebst and was produced by immunization with β-galactosidase−*psbA* fusion protein.

RESULTS

Verification of Mutant Genotypes. As discussed above, we have chosen to alter conserved, basic residues residing within the large extrinsic loop of CP 43 as initial sites for site-directed mutagenesis. For the mutants described herein, mutagenic olignucleotides with the sequences 5′-AAGTGG AAGGACCACAGGCTGGTTGCCAATCCA-3′ and 5′-CCGCCAGTTTGGTACAGA AAGACCCTGAAGGCA-3′ were used, respectively, to convert the arginine residues at positions 305 and 342 in the amino acid sequence of CP 43 to serine residues as described above. *Synechocystis* 6803 was transformed with the pTZ*psbC* phagemids bearing the desired site-directed mutations. After several rounds of streaking to allow sorting out of the mutations, kanamycin-resistant *Synechocystis* colonies were isolated, their genomic DNA was extracted, and the 2.4 kb *Acc*I/*Acc*I fragments were amplified by PCR. Amplified fragments were cloned into the pGEM-T vector (Promega). DNA sequencing (data not shown) confirmed the presence of the desired mutations. Additionally, the entire *Acc*I/*Acc*I fragment was sequenced and no other mutations were found to be present.

To ensure that the resulting phenotypes of the site-directed mutant strains resulted from the introduced mutations, rather than an unidentified second-site mutation(s), each site-directed mutant strain was transformed with the nonmutagenized pTZ*psbC* phagemid in order to convert the serine residues back to the original arginines. This should restore the same phenotype as the control strain. The genotype of these revertant strains was confirmed by sequencing the entire PCR-amplified *Acc*I/*Acc*I fragment from each (data not shown).

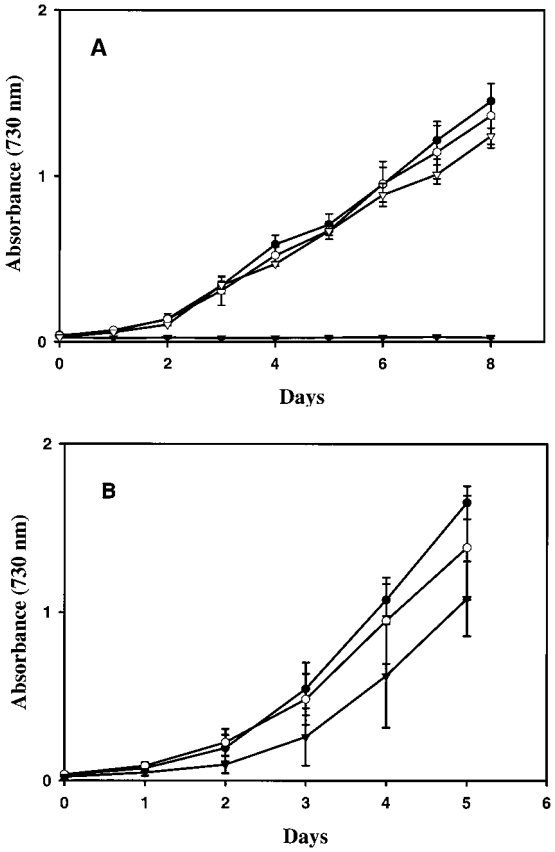

FIGURE 1: Growth characteristics of the control strain, site-directed mutants R305S and R342S, and the R342Sr revertant strain. (A) Photoautotrophic growth of control cells (○), R305S cells (●), R342S cells (▼), and R342Sr (▽) cells. (B) Mixotrophic growth of control cells (○), R305S (●), and R342S (▼) cells. These data represent the average of three independent experiments. The error bars are plus and minus 1 standard deviation.

Growth Characteristics. Figure 1 documents the results of growth experiments performed on the control strain, R305S and R342S mutant strains, and the R342Sr revertant strain. The control is a strain of *Synechocystis* that contains a kanamycin resistance gene in the 3′ flanking region of the *psbC* gene but lacks any site-directed alterations. Figure 1A documents the photoautotrophic growth of these cell lines in liquid BG-11 medium. R305S mutant cells grew at a rate comparable to controls and at rates identical to the R305Sr strain (data not shown). The growth of R342S cells differed dramatically from that of R305S and the control cells. This mutant exhibited no capacity for photoautotrophic growth. Restoration of the original arginine at position 342 in the strain R342Sr produced a strain with restored photoautotrophic growth at rates comparable to the control strain.

The mixotrophic growth characteristics of these cell lines in BG-11 medium are documented in Figure 1B. As expected, R305S mutant cells and control cells all grew at nearly identical maximal rates in BG-11 medium supple-

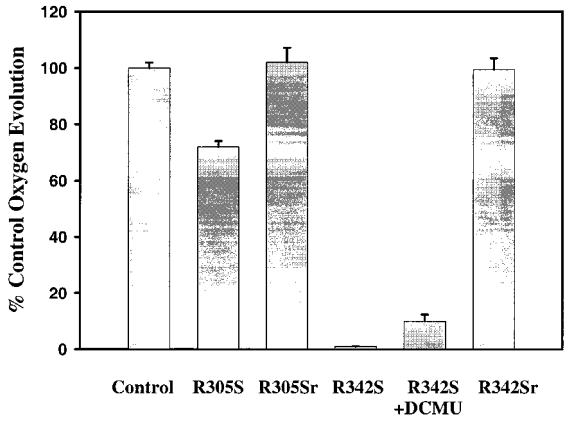

FIGURE 2: Oxygen evolution (water to DCBQ) of the control strain, site-directed mutants R305S and R342S, and the R305Sr and R342Sr revertant strains. Cells were grown in liquid BG-11 medium containing 5 mM glucose without DCMU, except where noted. The data represents the average of three independent experiments. The error bars are plus and minus 1 standard deviation. Oxygen evolution rates for the control strain averaged 545 μmol of O_2·(mg of chl)$^{-1}$·h^{-1}.

mented with 5 mM glucose. The R305Sr strain also grew at the same rate as the control and R305S mutant (data not shown). Growth of the R342S mutant strain, which failed to grow photoautotrophically, was restored in BG-11 medium supplemented with 5 mM glucose. The rate of growth of R342S cells was only slightly lower than that of controls but exhibited approximately a 1-day lag. As expected, the R342Sr strain also grew photomixotrophically at control rates (data not shown).

PSII Characterization. Figure 2 shows the steady-state oxygen evolution rates (measured as H_2O to DCBQ electron transport) observed for the control, mutant, and revertant cells grown in BG-11 medium in the presence of 5 mM glucose. The R305Sr and R342Sr strains exhibited 100% of the oxygen-evolving activity of controls. Oxygen evolution rates for the control strain averaged 545 μmol of O_2·(mg of chl)$^{-1}$·h^{-1}. The mutant strain R305S exhibited a 30% decrease in ability to evolve oxygen compared to the control. A much more dramatic effect on oxygen evolution rates was seen in the mutant R342S. This mutant exhibited essentially a zero rate of oxygen evolution when grown in BG-11 medium containing 5 mM glucose. Interestingly, when R342S cells were grown in glucose-containing medium to which DCMU had been added, some oxygen-evolving activity was observed. However, these rates were low, being approximately 10% of control rates.

Figure 3 illustrates the results from the immunological analysis of several PSII core components (CP 47, CP 43, D1, and the 33 kDa manganese-stabilizing protein). It is apparent that both R305S and R342S mutants contain very similar quantities of these proteins. It is important to note that these results are qualitative in nature and that minor differences in the amounts of these antigens would not be detected in this experiment.

To assess the PSII content of these mutants, variable fluorescence yields were measured for R305S and R342S along with the control strain and a *psbC* deletion strain (Table 1). Variable fluorescence yield can provide a semiquantitative estimate of the number of PSII centers that can utilize either water or hydroxylamine as an electron donor (*22*, *23*). It has

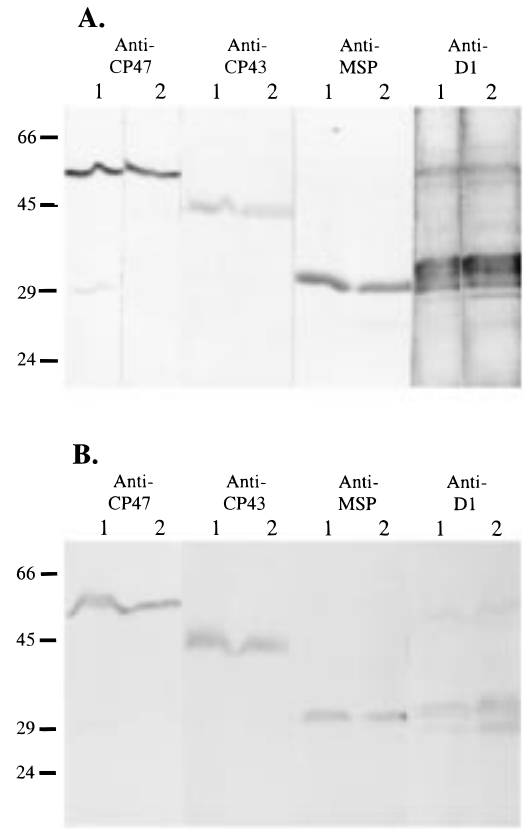

FIGURE 3: Western blots of control, R305S, and R342S thylakoid membrane proteins from cells grown in BG-11 medium supplemented with 5 mM glucose. Membrane proteins were separated by LDS−PAGE, electroblotted onto nitrocellulose, and, following blocking, probed with various primary antibodies. The protein bands were visualized by incubation with alkaline phosphatase-conjugated secondary antibodies and color development with bromochloroindoyl phosphate and nitroblue tetrazolium. Panel A, control (lane 1) and R305S (lane 2) proteins. Panel B, control (lane 1) and R342S (lane 2) proteins. The primary antibodies are indicated above.

Table 1: Variable Fluorescence Yields for Control and Site-Directed Mutant Strains

	cell type[a,b]			
	control	R305S	R342S	Δ*psbC*
HA → Q_A	0.53 (0.09)[c]	0.37 (0.11)	0.32 (0.05)	0.06 (0.02)
$t_{1/2}$[d] (ms)	13.5 (5.0)	15.7 (5.0)	19.4 (4.0)	nd[e]
H_2O → Q_A	0.35 (0.06)	0.38 (0.04)	0.15 (0.07)	0.02 (0.02)
$t_{1/2}$ (ms)	5.6 (0.9)	13.8 (6.0)	44.5 (19.0)	nd

[a] Cells were grown in liquid BG-ll medium supplemented with 5 mM glucose and 10 μM DCMU. [b] The data represent the average of two independent experiments. [c] Standard deviation is given in parentheses. [d] Fluorescence rise time. [e] Not determined.

been noted that in the combined analysis of 21 D1 mutants (*23*) and eight deletion mutants in the large extrinsic loop of CP 47 (*28*), a strong correlation was observed between the relative concentration of PSII centers determined either by [^{14}C]DCMU binding or by variable fluorescence yield. Our results indicate that the deletion strain contains essentially no functional PSII centers. Both R305S and R342S assemble similar numbers of PSII centers that can transfer electrons from hydroxylamine to Q_A. These mutants assemble 60−70% of the PSII centers assembled in the control strain. If water is used as an electron donor, the variable fluorescence yield is a measure of the amount of PSII centers that can oxidize water. The mutant R305S contains about the

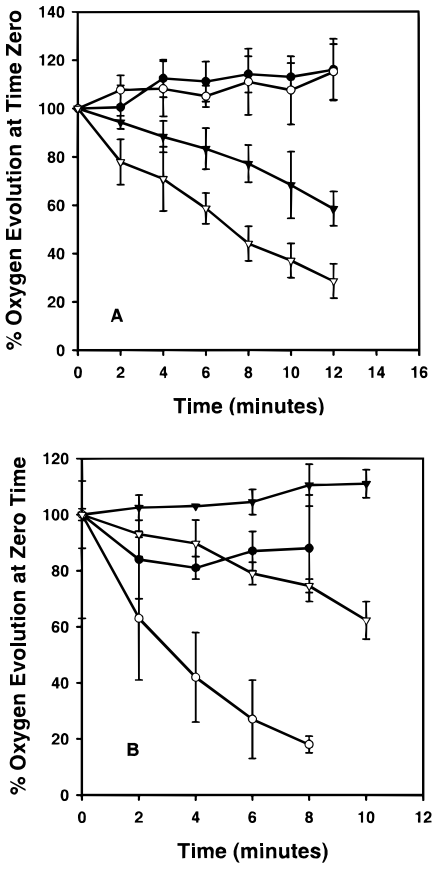

FIGURE 4: Photoinactivation experiment performed on the control strain and the site-directed mutants R305S and R342S at 5000 μmol of photons·m^{-2}·s^{-1}. (A) Control, no photoinactivating light (○); R305S, no photoinactivating light (●); control plus photoinactivating light (▼); R305S plus photoinactivating light (▽). (B) Control, no photoinactivating light (▼); R342S, no photoinactivating light (●); control plus photoinactivating light (▽); R342S plus photoinactivating light (○). The data are the average of three independent experiments. The error bars are plus and minus 1 standard deviation. The $t_{1/2}$ values were calculated from a fit of the data to a single-exponential decay. It was not possible to measure rates for R342S at the 10 min time point since we were at the limit of detection in our assay.

same number of PSII centers than can oxidize water as the control, while the mutant R342S contains about 40% of these centers.

It could be hypothesized that, while the mutants contain fewer functional PSII centers than the control strain, the centers that are present function normally. The results in Figure 4 indicate that this is not the case. Cells from each mutant and the control were subjected to photoinactivation at a light intensity of 5000 μmol of photons·m^{-2}·s^{-1} for various times and then PSII activity (H_2O to DCBQ) was measured. The calculated $t_{1/2}$ for photoinactivation for the R305S mutant (Figure 4A) was 2.7 times that of control cells, indicating that the mutant is more sensitive to photoinactivation than the control. The photoinactivation was difficult to measure in the R342S mutant given its low rate of oxygen evolution. Given this, R342S cells demonstrated an extreme sensitivity to photoinhibition, with a calculated $t_{1/2}$ for photoinactivation 4 times that of control (Figure 4B).

The fluorescence rise times, with both hydroxylamine and water as electron donors, were also measured. These results (Table 1) also indicated that the mutant reaction centers do not function normally. While the fluorescence rise times of the mutants were slightly slower than the control strain when hydroxylamine was used as an electron donor, large changes in the fluorescence rise time were observed when water was used as the electron donor. The fluorescence rise time of the mutant R305S was 2.5 times slower and the mutant R342S was nearly 8 times slower than that observed for the control strain.

DISCUSSION

Alteration of conserved basic residues in the large extrinsic loop of CP 43 was undertaken to test the hypothesis that these residues may be required for normal PSII activity. Two mutants, R305S and R342S, were constructed in *Synechocystis* by transformation and homologous recombination of mutant pTZ*psbC* phagemids. DNA sequencing of genomic DNA from each mutant confirmed the mutant genotypes. To additionally ensure that the mutant phenotypes resulted from the introduced mutations rather than mutations at distant sites, each mutant was transformed with the nonmutagenized pTZ*psbC* phagemid. These resulting revertants, R305Sr and R342Sr, both exhibited a phenotype indistinguishable from the control strain with respect to photoautotrophic growth and ability to evolve oxygen (Figures 1 and 2). These results demonstrate that the observed phenotypes of R305S and R342S are correlated with the introduction of the arginine to serine mutations.

The two mutants differed significantly in their growth characteristics. R305S cells grew photoautotrophically at rates identical to the control strain (Figure 1A). Many PSII mutants have been identified that show moderate alterations in the ability to evolve oxygen but grow well photoautotrophically. This is true for the RR384385GG mutant, which bears its mutation in the large extrinsic loop of CP 47; it is impaired in its ability to bind the manganese-stabilizing protein and exhibits decreased rates of oxygen evolution (*29*, *30*). Several deletion mutations in CP 47 that are impaired in oxygen-evolving activity also grow photoautotrophically at control rates (*31*, *32*). Unlike R305S, R342S failed to exhibit any ability to grow photoautotrophically (Figure 1A). Growth was only observed when cells were cultured in the presence of glucose (Figure 1B). The photoheterotrophic growth of *Synechocystis* in the absence of a functional PSII is well-documented (*21*).

The two mutants also differed in their abilities to support PSII-dependent (H_2O to DCBQ) oxygen evolution. R305S cells exhibited 70% of the oxygen-evolving activity of control cells (Figure 2). The mutant R342S exhibited an extremely low rate of oxygen-evolving (H_2O to DCBQ) activity, only 10% of that observed for control cells. Additionally, this low rate was only observed for cells grown photoheterotrophically in the presence of 10 μM DCMU (discussed below). Results of an immunological analysis of isolated thylakoid membranes from the two mutants failed to detect any differences in the levels of CP 47, CP 43, D1, or the manganese-stabilizing protein between mutant and control strains (Figure 3). Variable fluorescence yield measurements (hydroxylamine to Q_A) also indicated that the mutants assembled a significant amount of PSII reaction centers (60−70% of control) (Table 1).

While these mutants appear to assemble a significant amount of PSII, it is clear that the assembled PSII reaction

centers do not function normally. A number of lines of evidence support this contention. First, as shown in Figure 4, the H_2O to DCBQ electron transport activities in both mutants were more susceptible to photoinactivating radiation than the control strain. R305S and R342S cells exhibited $t_{1/2}$ values for photoinactivation that were, respectively, 2.7 and 4 times greater than that observed for control cells. The increased susceptibility to photoinactivation demonstrated by these mutants is on the order of that observed for the E69Q and P161L mutants in the D1 protein of *Synechocystis* and for the RR384385GG, RR384385EE, R448G, and K321G mutants in the CP 47 protein of *Synechocystis*. The E69Q mutation appears to affect the stability and/or ligation of the manganese cluster, while the P161L mutation appears to affect the efficiency of electron transfer from the oxygen-evolving site to Z^+ (*24*). The RR384385GG and RR384385EE mutants are impaired in their ability to bind the manganese-stabilizing protein and have an extended S_2 lifetime and a retarded rate of oxygen release (*30*). Both R448G and K321G mutants are believed to alter a chloride-binding site in PSII (*33*, *34*). For all of these mutants is has been speculated that the observed decreased rates of electron transfer from the oxygen-evolving complex to Z^+ resulted from accumulation of oxidizing-side radicals such as Z^+ and $p680^+$, which damage the reaction center and lead to photoinactivation. A similar mechanism may occur in the R305S and R342S mutants described here. The apparent protective effect on oxygen-evolving activity seen with the R342S mutant grown in the presence of DCMU may result from a reduction of damaging oxidizing-side radicals in the presence of this PSII inhibitor (*35*, *36*).

Second, the mutants exhibit a significant alteration in their fluorescence rise time characteristics. When hydroxylamine is used as an electron donor to PSII, the mutants exhibit a slightly slower rise from F_0 to F_{max} than we observed in the control strain. This indicates that there is only a slight perturbation in the electron transfer from Z (the donation site of hydroxylamine) to Q_A. When water was used as an electron donor, however, a large difference was observed. The fluorescence rise of the R305S mutant was slowed 2.5 times, while that of the R342S mutant was nearly 8 times slower than the control. These results indicate that electron flow from the oxygen-evolving complex is moderately retarded in R305S and severely retarded in R342S. It should be noted that these differences do not appear to be due to altered efficiency of excitation energy transfer to the reaction center. Such an alteration, while capable of slowing electron flow to Q_A, would be observed with both water and hydroxylamine as electron donors.

In the R342S mutant, the fluorescence yield with water as an electron donor is about 40% that observed in the control strain, while the steady-state oxygen evolution rate is severely depressed, being only 10% of that observed in the control. These observations could indicate that many (40%) the PSII reaction centers in R342S can oxidize water but that oxygen cannot be evolved efficiently. A number of hypotheses can be formed that could explain these observations. First, it is possible that a manganese cluster is present in R342S and that this manganese cluster can carry out the $S_1 \rightarrow S_2$ transition. If the recombination rate between Q_A^- and the S_2 state was accelerated, however, this could result in a slower rise to F_{max} and/or an overall decrease in the fluorescence yield. It is also possible, although unlikely, that the $S_1 \rightarrow S_2$ transition is dramatically slowed in this mutant. A second possibility is that most of the PSII reaction centers in R342S lack a functional manganese cluster. Under these conditions, alternative electron donors may become oxidized by Y_Z (*37*). If these electron donors were less efficient in electron donation than the oxygen-evolving complex, then one would expect a slower rise to F_{max}. It should be noted that hydroxylamine electron donation to Y_Z exhibits a similar effect in the control strain (Table 1), with the rise to F_{max} being slowed by a factor of about 2.4 when compared to electron donation by the oxygen-evolving site. A third possibility is that R342S exhibits hyperphotoinactivation. As demonstrated in Figure 4, R342S photoinactivates at a much greater rate than either the control strain or R305S. It is possible that, under steady-state oxygen evolution conditions, individual reaction centers can only carry out a few Kok cycles prior to photoinactivation. Given the low time resolution of the Clark-type polarographic electrode, many centers may be completely inactivated prior to the observation of any oxygen evolution signal. In the fluorescence experiment that was performed in the presence of DCMU, individual reaction centers only undergo the $S_1 \rightarrow S_2$ transition and such photoinactivation would not be observed. It should be noted that this hypothesis does not account for the slow fluorescence rise time observed in this mutant. At this point in time it is impossible to differentiate between these (and other) possible hypotheses and these are topics of further investigation.

What is the basis of the effects of these mutations on PSII? One possibility is that the association of the manganese-stabilizing protein is impaired in these mutants. Enami et al. (*16*) digested spinach PSII membranes with trypsin in the presence and absence of the manganese-stabilizing protein. They found that the residue ^{357}R in CP 43 was cleaved in the absence of the manganese-stabilizing protein but not in its presence. This residue is the equivalent of ^{342}R in *Synechocystis* CP 43. These authors speculated that a domain at or close to this site could serve as a binding site for the manganese-stabilizing protein. If the association of the manganese-stabilizing protein with PSII is altered, the phenotype of the mutants should resemble that of Δ*psbO* mutants (*1*, *3*). In these strains, constructed by deletion or insertional inactivation of the *psbO* gene that encodes the manganese-stabilizing protein, the mutants grew photoautotrophically but at lowered rates, appeared to accumulate normal levels of PSII proteins, and evolved oxygen at 40−70% of control rates. While the phenotype of R305S closely resembles that of the Δ*psbO* strains, the phenotype of R342S appears to be significantly more severe and cannot be explained by an alteration in manganese-stabilizing protein binding alone. Further studies are underway to elucidate the nature of the oxygen evolution defects in both of these mutants.

CONCLUSIONS

The results presented here strengthen the hypothesis that CP 43 interacts with the oxygen-evolving site of PSII. This is the first study utilizing site-directed mutagenesis that suggests a possible role for CP 43 in water oxidation. The substitution of serine for arginine at positions 305 and 342 in the large extrinsic loop of CP 43 resulted in mutants

showing reduced rates of steady-state oxygen evolution, enhanced rates of photoinactivation, and decreased numbers of fully functional PSII centers. These results are consistent with the hypothesis that these alterations each introduce a defect at the oxygen-evolving site of PSII. The exact nature of the lesion introduced with the oxygen-evolving complex remains to be defined.

REFERENCES

1. Burnap, R. L., and Sherman, L. A. (1991) *Biochemistry 30*, 440−446.
2. Bricker, T. M. (1992) *Biochemistry 31*, 4623−4628.
3. Philbrick, J. B., Diner, B. A., and Zilinskas, B. A. (1991) *J. Biol. Chem. 266*, 13370−13376.
4. Shen, J.-R., and Inoue, Y. (1993) *Biochemistry 32*, 1825−1832.
5. Debus, R. J. (1992) *Biochim. Biophys. Acta 1102*, 269−352.
6. Chisholm, D., and Williams, J. G. K. (1988) *Plant Mol. Biol. 10*, 293−301.
7. Bricker, T. M. (1990) *Photosynth. Res. 24*, 1−13.
8. Sayre, R. J., and Wrobelboerner, E. A. (1994) *Photosynth. Res. 40*, 11−19.
9. Bricker, T. M., and Ghanotakis, D. (1996) in *Oxygenic Photosynthesis: The Light Reactions* (Yocum, C. F., and Ort, D. R., Eds.) pp 113−136, Kluwer Academic Publishers, Dordrecht, The Netherlands.
10. Rochaix, J.-D., Kuchka, M., Mayfield, S., Shirmer-Rahire, M., Girard-Bascou, J., and Bennoun, P. (1989) *EMBO J. 8*, 1013−1021.
11. Carpenter, S. D., Charite, J., Eggers, B., and Vermaas, W. F. (1990) *FEBS Lett. 260*, 135−137.
12. Rogner, M., Chisholm, D. A., and Diner, B. A. (1991) *Biochemistry 30*, 5387−5395.
13. Vermaas, W. F. J., Ikeuchi, M., and Inoue, Y. (1988) *Photosynth. Res. 17*, 97−113.
14. Dzelzkalns, V. A., and Bogorad, L. (1988) *EMBO J. 7*, 333−338.
15. Kuhn, M. G., and Vermaas, W. F. (1993) *Plant Mol. Biol. 23*, 123−133.
16. Enami, I., Satoh, K., Tohri, A., Kamo, M., Ohta, H., and Shen, J.-R. (1997) *Biochim. Biophys. Acta 1320*, 17−26.
17. Rippka, R., Derulles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. (1979) *J. Gen. Microbiol. 111*, 1−61.
18. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning*: *A Laboratory Manual*, 1st ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
19. Goldfarb, N. E., Knoepfle, N., and Putnam-Evans, C. (1997) *SAAS Bull. Biochem. Biotechnol. 10*, 1−6.
20. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
21. Williams, J. G. K. (1988) *Methods Enzymol. 167*, 766−778.
22. Nixon, P., and Diner, B. (1992) *Biochemistry 31*, 942−948.
23. Chu, H.-A., Nguyen, A. P., and Debus, R. J. (1994) *Biochemistry 33*, 6137−6149.
24. van der Bolt, F., and Vermaas, W. F. J. (1992) *Biochim. Biophys. Acta 1098*, 247−254.
25. Bricker, T. M., Odom, W. R., and Queirolo, C. B. (1988) *FEBS Lett. 231*, 111−117.
26. Pratt, L. H., McCurdy, D. W., Shimazaki, Y., and Cordonnier, M.-M. (1986) in *Modern methods of plant analysis* (Linskens, H. F., and Jackson, J. F., Eds.) pp 50−74, Springer-Verlag, New York.
27. Chua, N.-H., and Bloomberg, F. (1979) *J. Biol. Chem. 254*, 215−223.
28. Gleiter, H. M., Haag, E., Shen, J. R., Eaton-Rye, J. J., Inoue, Y., and Vermaas, W. F. J. (1994) *Biochemistry 33*, 12063−12071.
29. Putnam-Evans, C., and Bricker, T. M. (1992) *Biochemistry 31*, 11482−11488.
30. Putnam-Evans, C., Wu, J., Burnap, R., Whitmarsh, J., and Bricker, T. M. (1996) *Biochemistry 35*, 4046−4053.
31. Haag, E., Eaton-Rye, J. J., Renger, G., and Vermaas, W. F. J. (1993) *Biochemistry 32*, 4444−4454.
32. Eaton-Rye, J. J., and Vermaas, W. F. J. (1991) *Plant Mol. Biol. 17*, 1165−1177.
33. Putnam-Evans, C., and Bricker, T. M. (1994) *Biochemistry 33*, 10770−10776.
34. Putnam-Evans, C., and Bricker, T. M. (1997) *Plant Mol. Biol. 34*, 455−463.
35. Wu, J., Putnam-Evans, C., and Bricker, T. M. (1996) *Plant Mol. Biol. 32*, 537−542.
36. Chu, H.-A., Nguyen, A. P., and Debus, R. J. (1995) *Biochemistry 34*, 5859−5882.
37. Miller, A.-F., and Brudvig, G. W. (1990) *Biochemistry 29*, 1385−1392.

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