Site-Directed Mutagenesis of Glutamate Residues in the Large Extrinsic Loop of the Photosystem II Protein CP 43 Affects Oxygen-Evolving Activity and PS II Assembly†

Christina Rosenberg,‡ Julie Christian,‡ Terry M. Bricker,§ and Cindy Putnam-Evans*,‡

Department of Biology, East Carolina University, Greenville, North Carolina 27858, and Department of Biological Sciences, Biochemistry and Molecular Biology Section, Louisiana State University, Baton Rouge, Louisiana 70803

Received June 10, 1999; Revised Manuscript Received October 5, 1999

ABSTRACT: The *psb*C gene encodes the intrinsic chlorophyll protein CP 43, a component of photosystem II in higher plants, green algae, and cyanobacteria. Oligonucleotide-directed mutagenesis was used to introduce mutations into the portion of *psb*C that encodes the large extrinsic loop E of CP 43 in the cyanobacterium *Synechocystis* 6803. Three mutations, E293Q, E339Q, and E352Q, each produced a strain with impaired photosystem II activity. The E293Q mutant strain grew photoautotrophically at rates comparable to the control strain. Immunological analyses of several PS II components indicated that this mutant accumulated normal quantities of PS II proteins. However, this mutant evolved oxygen to only 56% of control rates at saturating light intensities. Measurements of total variable fluorescence yield indicated that this mutant assembled approximately 60% of the fully functional PS II centers found in the control strain. The E339Q mutant grew photoautotrophically at a severely reduced rate. Both immunological analysis and variable fluorescence yield experiments indicated that E339Q assembled a normal complement of PS II centers. However, this mutant was capable of evolving oxygen to only 20% of control rates. Variable fluorescence yield experiments demonstrated that this mutant was inefficient at using water as an electron donor. Both E293Q and E339Q strains exhibited an increased (approximately 2-fold) sensitivity to photoinactivation. The E352Q mutant was the most severely affected. This mutant failed to grow photoautotrophically and exhibited essentially no capacity for oxygen evolution. Measurements of total variable fluorescence yield indicated that this mutant assembled no functional PS II centers. Immunological analysis of isolated thylakoid membranes from E352Q revealed a complete absence of CP 43 and reduced levels of both the D1 and manganese-stabilizing proteins. These results suggest that the mutations E293Q and E339Q each produce a defect associated with the oxygen-evolving complex of photosystem II. The E352Q mutation appears to affect the stability of the PS II complex. This is the first report showing that alteration of negatively charged residues in the CP 43 large extrinsic loop results in mutations affecting PS II assembly/function.

The photosystem II (PS II)[1] complex catalyzes the light-dependent oxidation of water and concomitant reduction of plastoquinone to plastoquinol. Both intrinsic and extrinsic protein components comprise this multisubunit protein−pigment complex. The intrinsic components associated with the thylakoid membrane that are absolutely required for the formation of a PS II complex capable of evolving 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 *psb*C gene, CP 43 is an integral thylakoid protein and component of the proximal antennae of PS II (*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}Trp 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*). CP 43 functions as an interior chlorophyll-*a*

† This work was sponsored by National Science Foundation grants to C.P.-E. and T.M.B. and by a U.S. Department of Energy grant to T.M.B.

* Correspondence should be addressed to this author at the Department of Biology, East Carolina University, Greenville, NC 27858. Telephone: (252) 328-4811. FAX: (252) 328-4178. Email: evansc@mail.ecu.edu.

‡ East Carolina University.

§ Louisiana State University.

[1] Abbreviations: PS II, photosystem II; kb, kilobase pair(s); 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, kilodalton(s); LDS, lithium dodecyl sulfate; HA, hydroxylamine.

10.1021/bi991326r CCC: $18.00
Published on Web 11/03/1999

light-harvesting antennae for PS II. However, accumulating data also support a role for CP 43 in the stable assembly of PS II and in the oxygen-evolving process. CP 43 is a component of all oxygen-evolving PS II preparations that have been isolated (*9*). *Chlamydomonas* mutants bearing alterations in *psb*C that affect either the synthesis or the stability of CP 43 were deficient in PS II activity (*10*). In these mutants, the levels of other PS II core proteins within the thylakoid were severely reduced. Deletion of *psb*C in *Synechocystis* produced mutants that accumulated PS II core complexes to only 10% of wild-type levels. These mutants could not grow photoautotrophically or evolve oxygen (*11, 12*). Though these core complexes lacked CP 43, electron transport from Z to Q_A was supported (*12*). However, electron transport from Q_A to Q_B was impaired, and this was suggested to account for the loss of oxygen-evolving activity. Interruption of the *psb*C gene by insertional mutagenesis also produced a mutant incapable of evolving oxygen, but which exhibited primary charge separation (*13*). Following nitrosoguanidine mutagenesis, Dzelzkalns and Bogorad (*14*) recovered a *Synechocystis* mutant unable to evolve oxygen or support PS II electron transport from water to either dichlorobenzoquinone (DCBQ) or methyl viologen. Further characterization of this mutant showed that it contained a short deletion within the *psb*C gene. Additionally, isolated thylakoid membranes from this mutant contained decreased levels of the reaction center protein D2. These data collectively suggest that CP 43 is essential for normal PS II assembly and function.

Eight short deletions were introduced within the large extrinsic loop E of CP 43 in *Synechocystis* by Kuhn and Vermaas (*15*). Significantly, all resulting mutants showed complete loss of photoautotrophic growth and the ability to evolve oxygen. These mutants also contained decreased levels of the PS II 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 PS II function. The loop contains a number of conserved charged amino acid residues (*7*), which may provide sites of interaction between CP 43 and the manganese-stabilizing protein or other protein components or ionic cofactors. Site-directed mutagenesis of positively charged arginine residues at sites 305 and 342 in the CP 43 large extrinsic loop produced mutants that exhibited decreased rates of steady-state oxygen evolution, enhanced rates of photoinactivation, and decreased numbers of fully functional PS II centers (*17*). In this report, we show that alteration of the acidic residues ^{293}E, ^{339}E produced mutants with impaired PS II activity associated with a defect at the oxygen-evolving site of PS II. Alteration of ^{352}E resulted in a mutant strain defective in PS II assembly.

EXPERIMENTAL PROCEDURES

Growth Conditions. Wild-type and mutant *Synechocystis* sp. PCC 6803 were grown in liquid BG-11 media (*18*) at 30 °C and at a light intensity of 25 μmol of photons·m^{-2}·s^{-1}. Cultures were shaken on a rotary shaker at 200 rpm. Where appropriate, glucose was added to cultures at 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 media to a final concentration of 10 μg/mL. Cultures maintained on plates in BG-11 media were supplemented with 1.5% agar, 0.3% sodium thiosulfate, 10 mM TES/KOH, pH 8.2, 10 μM DCMU.

Site-Directed Mutagenesis. Standard procedures (*19*) were employed for restriction digests, cloning, growth and transformation of bacterial strains, and isolation of DNA fragments. Phagemid isolations were performed using disposable anion-exchange columns (Promega, Inc.). Construction of the pTZ*psb*C phagemid used for mutagenesis was as described in (*20*). Briefly, a 2.4 kb *Acc*I/*Acc*I fragment of *psb*C 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 (BioRad, Inc.). This fragment contained the entire large extrinsic loop coding region as well as 0.32 kb of *psb*C 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 *psb*C coding region. Transformation of wild-type *Synechocystis* with this phagemid, designated pTZ*psb*C, resulted in the kanamycin-resistant control strain which contained an intact *psb*C gene, but no site-directed mutations. Desired mutations were introduced into pTZ*psb*C by oligonucleotide-directed mutagenesis using the procedure of Kunkel et al. (*21*). For the mutants described herein, mutagenic olignucleotides with the sequences 5′-CTTTGGGATTGGGAGGC**CTG**CATGCCGGTGGGGCC-3′, 5′-CCAGAAACGCATGGTG**CTG**ACCGCCGCCGAAGATGAT-3′, and 5′-GGGTCCACGGAGGGG**CTG**CAACCAAGGACCACGGAA-3′ were used, respectively, to convert the glutamate residues at positions 293, 339, and 352 in the amino acid sequence of CP 43 to glutamine residues. A *Synechocystis* 6803 *psb*C deletion strain (*20*) was transformed with the pTZ*psb*C phagemids bearing the desired site-directed mutations. Transformations were carried out by the procedure of Williams (*22*). Following a period of streaking individual colonies to allow sorting out of mutations, colonies of putative site-directed mutants were screened for kanamycin resistance. These colonies were streaked on kanamycin-containing plates in the presence or absence of glucose to screen for possible loss of photoautotrophic growth.

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 (*22*) except that the cesium chloride steps were omitted. Oligonucleotides flanking the *Acc*I/*Acc*I fragment of the *psb*C gene were used to amplify this region of the genomic DNA of each mutant using the polymerase chain reaction. The thermal cycling routine consisted of the following steps performed on 52 μL reactions: 1 min denaturation at 94 °C, 40 s denaturation at 92 °C, 40 s annealing at 45 °C, and 1.5 min 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 using 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). DNA sequencing confirmed the presence of the desired mutations

(data not shown). Additionally, the entire *Acc*I/*Acc*I fragment was sequenced, and no other mutations were found to be present.

Oxygen Evolution Assays. PS II activity was measured by O_2 polarography with a Hansatech oxygen electrode. Assays were performed at 25 °C on whole cells in BG-11 media using 1 mM DCBQ as an electron acceptor. The light intensity for these experiments was 2500 μmol of photons·m^{-2}·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 media at a chlorophyll concentration of 10 μg/mL at 5000 μmol of photons·m^{-2}·s^{-1} at 25 °C. At the indicated times, aliquots were removed and assayed for oxygen-evolving activity as described above. For the light saturation experiments, the light intensity was varied between 25 and 100 μmol of photons·m^{-2}·s^{-1}. The chlorophyll concentration in all oxygen evolution assays was 10 μg/mL. Chlorophyll content was measured as in Williams (*22*).

Fluorescence Measurements. Fluorescence yield measurements were performed on a Walz PAM 101 fluorometer as described previously (*23, 24*). Samples (10 μg/mL chlorophyll) were incubated in the dark for 5 min in the presence of 1 mM potassium ferricyanide and 330 μM DCBQ. DCMU was added to a final concentration of 40 μM followed 1 min 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·m^{-2}·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, $F_{v(H2O)}$, 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 (*25*). These membranes were subjected to electrophoresis on LDS−polyacrylamide gels, "Western blotted" onto nitrocellulose, and probed with antibodies to PS II core proteins essentially as described by Bricker et al. (*26*), 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 was with bromochloroindoyl phosphate and nitroblue tetrazolium as described by Pratt et al. (*27*). The anti-CP 43 antibody was a gift from Dr. N.-H. Chua and was raised against the purified polypeptide from *Chlamydomonas* (*28*). The anti-CP 47 antibody was raised against purified spinach CP 47 (Leuschner, C., Sherman, L., and Bricker, T. M., unpublished experiments). The anti-D1 antibody was supplied by Dr. A. Trebst and was produced by immunization with β-galactosidase−*psb*A fusion protein.

RESULTS

Growth Characteristics. Figure 1 documents the results of growth experiments performed on the control strain and the E293Q, E339Q, and E352Q mutant strains. The control is a strain of *Synechocystis* that contains a kanamycin resistance gene in the 3′ flanking region of the *psb*C gene but which lacks any site-directed alterations. Figure 1 documents the photoautotrophic growth of these cell lines in liquid BG-11 media without glucose. E293Q mutant cells grew at a rate comparable to controls. The growth of E339Q and E352Q cells differed dramatically from that of E293Q and the control cells. E339Q cells grew at a barely perceptible rate, while E352Q cells exhibited no capacity for photoautotrophic growth. The mixotrophic growth characteristics of these cell lines in BG-11 media supplemented with 5 mM glucose were also documented (data not shown). Growth was restored in the E339Q and E352Q mutants to rates comparable to the control growth rate. As expected, E293Q mutant cells grew at nearly identical maximal rates as the control under photomixotrophic conditions.

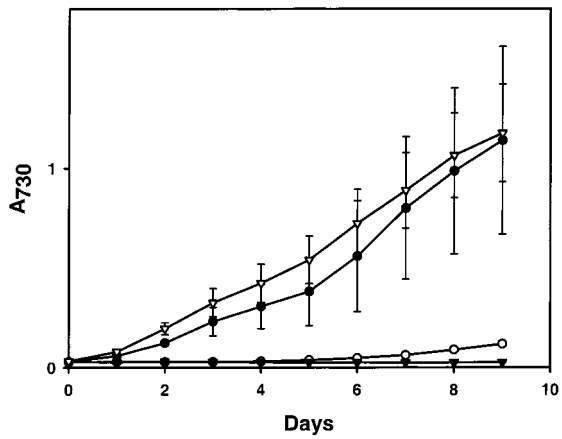

FIGURE 1: Photoautotrophic growth characteristics of the control strain (closed circles) and the site-directed mutants E293Q (open triangles), E339Q (open circles), and E352Q (closed triangles). Cells were grown in liquid BG-11 media without glucose, and growth was assessed by measuring the A_{730} of each culture daily. These data represent the average of three independent experiments. The error bars are plus and minus one standard deviation.

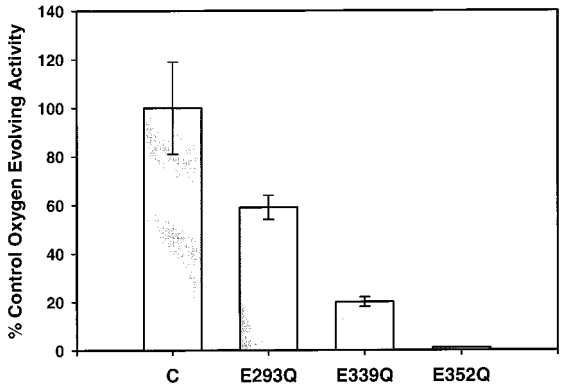

FIGURE 2: Oxygen evolution (water to DCBQ) of the control strain and site-directed mutants E293Q, E339Q, and E352Q. Cells were grown in liquid BG-11 media containing 5 mM glucose. The data represent the average of three independent experiments. The error bars are plus and minus one standard deviation. Oxygen evolution rates for the control strain averaged 506 μmol of O_2·(mg of chl)$^{-1}$·h^{-1}.

PS II Characterization. Figure 2 shows the steady-state oxygen evolution rates (measured as H_2O to DCBQ electron transport) observed for the control and mutant strains grown in BG-11 media in the presence of 5 mM glucose. Oxygen evolution rates for the control strain averaged 506 μmol of O_2·(mg of chl)$^{-1}$·h^{-1}. The mutant strain E293Q exhibited a 44% decrease in ability to evolve oxygen compared to the

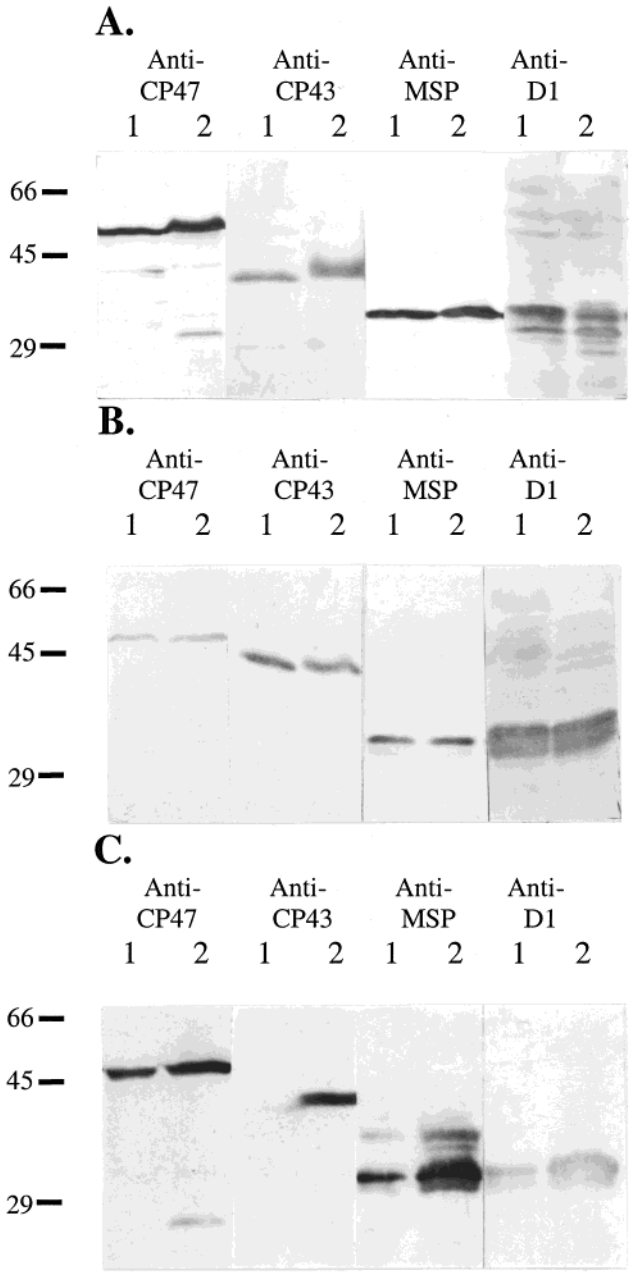

FIGURE 3: Western blots of control, E293Q, E339Q, and E352Q thylakoid membrane proteins from cells grown in BG-11 media supplemented with 5 mM glucose. Membrane proteins were separated by LDS−PAGE, electroblotted onto nitrocellulose, and, following blocking, probed with various primary antibodies. The protein bands were visualized by incubation with alkaline phosphatase conjugated secondary antibodies and color development with bromochloroindoyl phosphate and nitroblue tetrazolium. Panel A: E293Q (lane 1) and control (lane 2) proteins. Panel B: E339Q (lane 1) and control (lane 2) proteins. Panel C: E352Q (lane 1) and control (lane 2) proteins. The primary antibodies are indicated above.

control, while the E339Q mutant strain could evolve oxygen to only 20% of the control rate. A much more dramatic effect on oxygen evolution rates was seen in the mutant E352Q. This mutant exhibited essentially a zero rate of oxygen evolution when grown in BG-11 media containing 5 mM glucose.

Figure 3 illustrates the results from the immunological analysis of several PS II core components (CP 47, CP 43, D1, and the 33 kDa manganese-stabilizing protein). It is apparent that both the E293Q and E339Q mutants contain very similar quantities of these proteins. Due to the qualitative nature of these experiments, we would expect that minor differences in the amounts of these antigens could not be detected. Interestingly, the E352Q mutant is devoid of any detectable CP 43 protein in the thylakoid. Additionally, there appears to be a significant decrease in the amount of both D1 and the manganese-stablilizing protein in the thylakoids of this mutant.

Table 1: Characterization of Mutants

cell type	F_v,[a] $HA \rightarrow Q_A$	F_v, $H_2O \rightarrow Q_A$	Φ_{O2}[c]	photoinactivation rate[d]
control	0.63[b] (0.10)	0.46 (0.18)	1.00	1
E293Q	0.40 (0.09)	0.26 (0.02)	0.71	2
E339Q	0.61 (0.03)	0.20 (0.02)	0.12	2
E352Q	0.05 (0.02)	0.003 (0.003)	ND[e]	ND
ΔpsbC	0.05 (0.02)	0.003 (0.003)	ND	ND

[a] Cells were grown in liquid BG-11 media supplemented with 5 mM glucose and 10 μM DCMU. The data represent the average of two independent experiments. [b] Standard deviation. [c] Relative quantum yield. The data represent the average of three independent experiments. [d] The data represent the average of three independent experiments.[e] Not determined since these mutants were devoid of oxygen-evolving activity.

Measurement of oxygen evolution rates at limiting light intensities in the E239Q and E339Q mutant strains resulted in the quantum yield (Φ_{O2}) experiments summarized in Table 1. Analysis of the first-order rate constants for all strains indicated that the E293Q mutant had a Φ_{O2} of 0.71 and the E339Q mutant a Φ_{O2} of 0.12 with respect to the control strain. These data agree reasonably well with the observed steady-state oxygen evolution rates for these mutants compared to the control. The data suggest either (1) that the mutants contain a full complement of PS II centers that are damaged to such an extent that they cannot efficiently evolve oxygen or (2) that the mutants contain reduced numbers of PS II centers in the thylakoid that may or may not function normally. For the E339Q mutant, the former appears to be the case, and for the E293Q mutant, the latter appears correct (see below). This experiment could not be performed on the E352Q mutant since it failed to evolve oxygen.

To assess the PS II content of these mutants, variable fluorescence yields were measured for all three mutants along with the control strain and a *psb*C deletion strain (Table 1). Variable fluorescence yields provide a semiquantitative estimate of the number of PS II centers that can utilize either water or hydroxylamine as an electron donor (*23, 24*). It has been noted that in the combined analysis of 21 D1 mutants (*24*) and 8 deletion mutants in the large extrinsic loop of CP 47 (*29*), a strong correlation was observed between the relative concentration of PS II centers determined either by (1) [^{14}C]DCMU binding or by (2) variable fluorescence yield in the presence of exogenous electron donors. Our results indicate that the E293Q mutant strain assembles about 60% of PS II centers that can transfer electrons from hydroxylamine to Q_A compared to the control strain. This number is in good agreement with the data from steady-state oxygen evolution rates and the relative quantum yield. The E339Q mutant appears to assemble essentially the same number of PS II centers that can transfer electrons from hydroxylamine to Q_A as the control, though, as discussed above, it only evolves oxygen under steady-state conditions at 20% of the

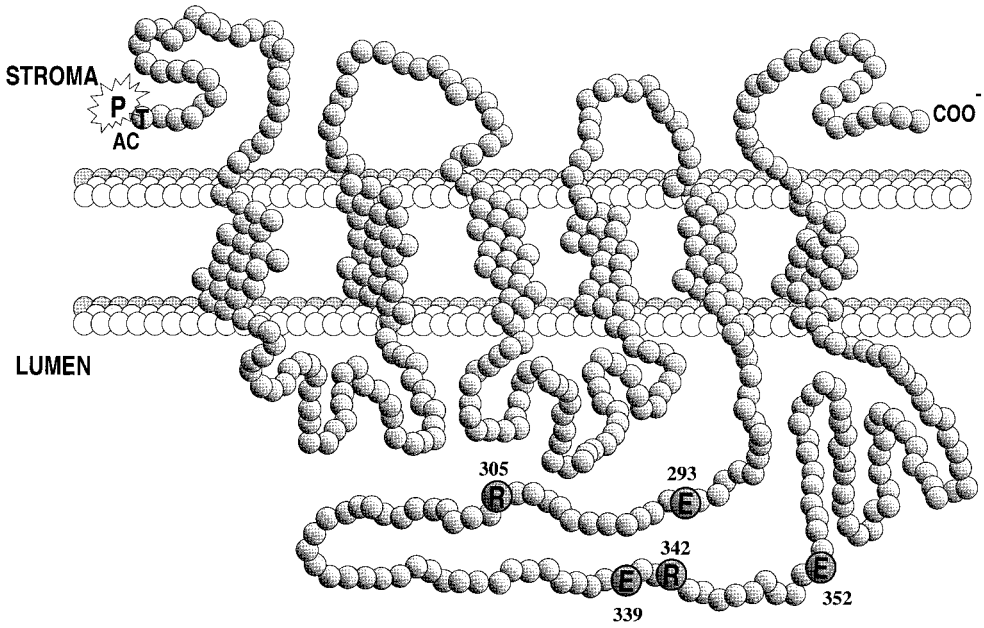

FIGURE 4: Model of the CP 43 protein from *Synechocystis* 6803. The positions of all the charged residues that we have changed to date that result in mutants with altered PS II function are indicated by the one-letter amino acid code for these residues. It should be pointed out that in our numbering of amino acids in CP 43 from *Synechocystis* we assume the translation start site is a valine 12 residues downstream of the methionine start site in spinach CP 43. Thus, the ^{293}E, ^{339}E, and ^{352}E residues in *Synechocystis* that are the subject of this study correspond to glutamates at positions 308, 354, and 367 in spinach CP 43.

control rate. Interestingly, the variable fluorescence yield for the E352Q mutant strain was identical to that of the *psb*C deletion strain that contains essentially no functional PS II centers, indicating that the E352Q mutant also fails to assemble functional PS II centers. If water is used as an electron donor, the variable fluorescence yield is a measure of the amount of PS II centers that can oxidize water. The data indicate that the mutant E293Q contains about 60% of the PS II centers capable of oxidizing water compared to the control, while the E339Q mutant contains about 40% of these centers. The variable fluorescence yield (H_2O to Q_A) for the E352Q mutant was the same as for the *psb*C deletion strain, as expected since this strain lacks functional PS II centers.

It could be hypothesized that, while the E293Q and E339Q mutants contain fewer functional PS II centers than the control strain, the centers that are present function normally. The results in Table 1 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$\cdot m^{-2}\cdot s^{-1}$ for various times, and then PS II activity (H_2O to DCBQ) was measured. The calculated $t_{1/2}$ for photoinactivation for both the E339Q and E352Q mutants was approximately 2 times that of control cells, indicating that both of these mutants are more sensitive to photoinactivation than the control. Again, it was not possible to perform this experiment on the E352Q mutant since it failed to evolve oxygen.

DISCUSSION

Site-directed mutagenesis of the conserved glutamate and aspartate residues in the CP 43 large extrinsic loop of *Synechocystis* was undertaken in order to test the hypothesis that acidic residues within this region are required for normal PS II function. Three mutations, E293Q, E339Q, and E352Q (Figure 4), resulted in strains with impaired PS II activity.

These mutants differed significantly in their growth characteristics (Figure 1). E293Q cells grew photoautotrophically at rates identical to the control strain. Many PS II mutants have been constructed that evolve oxygen at moderately reduced rates, but that grow well photoautotrophically (*30*−*33*), including the R305S mutant in the CP 43 large extrinsic loop (*17*). Our E339Q mutant was severely impaired in its ability to grow photoautotrophically. The maximal absorbance after 9 days of growth was only 10% that of the control culture. The E352Q mutant failed to exhibit any ability to grow photoautotrophically. Growth of E339Q and E352Q cells was only observed when cells were cultured in the presence of glucose (data not shown). The photoheterotrophic growth of *Synechocystis* in the absence of a functional PS II is well-documented (*22*).

The three mutants also differed in their abilities to support PS II-dependent (H_2O to DCBQ) oxygen evolution (Figure 2). E293Q cells exhibited approximately 60% of the oxygen-evolving activity of control cells. The mutant E339Q exhibited an even lower rate of oxygen-evolving (H_2O to DCBQ) activity, only 20% of that observed for control cells. The E352Q mutant failed to evolve oxygen.

Results of an immunological analysis of isolated thylakoid membranes from the E293Q and E339Q mutants failed to detect any differences in the levels of CP 47, CP 43, D1, or the manganese-stabilizing protein between these two mutants and the control strain (Figure 3). Variable fluorescence yield measurements (hydroxylamine to Q_A) indicated that the E293Q mutant assembled approximately 60% of PS II reaction centers found in the control, while the E339Q mutant assembled control numbers of PS II centers (Table 1).

While both of these mutants appear to assemble a significant amount of PS II, it is clear that the assembled PS II reaction centers do not function normally. As shown in Table 1, the H_2O to DCBQ electron transport activities in both mutants were more susceptible to photoinactivating

radiation than the control strain. E293Q and E339Q cells exhibited $t_{1/2}$'s for photoinactivation that were approximately 2 times greater than that observed for control cells. This increased susceptibility to photoinactivation is on the order of that observed for numerous mutants in the CP 43, CP 47, and D2 proteins of *Synechocystis.* The R305S and R342S mutants in the CP 43 protein affect the efficiency of electron transfer from the oxygen-evolving site to Z^+ (*17*). The E69Q mutation in the D2 protein appears to affect the stability and/or ligation of the manganese cluster while the P161L mutation in D2 appears to alter the efficiency of electron transfer from the oxygen-evolving site to Z^+ (*25*). The RR384385GG and RR384385EE mutants in CP 47 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 (*31*). Both the R448G and K321G mutants in CP 47 are believed to alter a chloride binding site in PS II (*34*, *35*). For all of these mutants, it 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 E293Q and E339Q mutants described here.

The E339Q mutant is extremely interesting. Analysis of its polypeptide composition and fluorescence yield (using HA as an electron donor) indicates that E339Q assembles a relatively normal number of PS II reaction centers. Its ability to evolve oxygen, however, is greatly compromised. Steady-state oxygen evolution rates (at saturating light intensities), quantum yield measurements for oxygen evolution, and fluorescence yield (using water as an electron donor) all indicate a defect in the ability of this mutant to carry out water oxidation. This is the first mutant described in CP 43 with these characteristics.

The most dramatic effect on PS II was observed in the E352Q mutant. This mutant failed to grow photoautotrophically and did not evolve oxygen (Figures 1 and 2). These results can be explained by the fact that this mutant does not assemble any detectable amount of functional PS II centers. Immunological analysis (Figure 3) showed that, of the PS II core proteins probed for in isolated thylakoids from this mutant, the CP 43 protein was completely absent. These mutant thylakoids additionally contained decreased levels of D1 and the manganese-stabilizing protein. The variable fluorescence yields (using either water or hydroxylamine as an electron donor) for the E352Q mutant and a Δ*psb*C deletion strain constructed in our laboratory were identical (Table 1). In this Δ*psb*C strain, the CP 43 coding sequence has been replaced by a spectinomycin resistance cartridge (*20*). This deletion strain fails to assemble PS II centers and does not evolve oxygen. These results demonstrate that the loss of function observed in the E352Q mutant is a result of the inability to assemble functional centers in the thylakoids of this mutant. These results are similar to those obtained by a number of investigators who have constructed mutants lacking CP 43 by either deletion mutagenesis (*12*) or insertional mutagenesis (*13*) of the *psb*C gene. These mutants, in which CP 43 is absent from the thylakoid, do not grow photoautotrophically or evolve oxygen. It is interesting that alteration of a single amino acid in CP 43 leads to the same catastrophic phenotype as observed in the deletion mutants. These results clearly demonstrate that CP 43 is essential for the formation of fully functional PS II centers.

CONCLUSIONS

The results presented strengthen the hypothesis that CP 43 interacts with the oxygen-evolving site of PS II, and this is the first report to indicate that negatively charged amino acid residues are important for this interaction. The substitution of glutamine for glutamate at positions 293 and 339 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 PS II centers. These results indicate that these mutations each introduce a defect at the oxygen-evolving site of PS II; however, the exact nature of the lesion remains to be defined. The introduction of glutamine at position 352 in place of glutamate produced a mutant that was unable to incorporate CP 43 into the thylakoid and thus could not form functional PS II centers. This is the first report of a single amino acid substitution within the CP 43 large extrinsic loop that leads to a mutation affecting assembly of the PS II complex.

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.
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. Knoepfle, N. K., Bricker, T. M., and Putnam-Evans, C. (1999) *Biochemistry 38*, 1582−1588.
18. Rippka, R., Derulles, J., Waterbury, J. B., Herdman, M., and Stanier, R. Y. (1979) *J. Gen. Microbiol. 111*, 1−61.
19. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning—A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
20. Goldfarb, N. E., Knoepfle, N., and Putnam-Evans, C. (1997) *SAAS Bull. Biochem. Biotechnol. 10*, 1−6.
21. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.

22. Williams, J. G. K. (1988) *Methods Enzymol. 167*, 766−778.
23. Nixon, P., and Diner, B. (1992) *Biochemistry 31*, 942−948.
24. Chu, H.-A., Nguyen, A. P., and Debus, R. J. (1994) *Biochemistry 33*, 6137−6149.
25. van der Bolt, F., and Vermaas, W. F. J. (1992) *Biochim. Biophys. Acta 1098*, 247−254.
26. Bricker, T. M., Odom, W. R., and Queirolo, C. B. (1988) *FEBS Lett. 231*, 111−117.
27. 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.
28. Chua, N.-H., and Bloomberg, F. (1979) *J. Biol. Chem. 254*, 215−223.
29. Gleiter, H. M., Haag, E., Shen, J.-R., Eaton-Rye, J. J., Inoue, Y., and Vermaas, W. F. J. (1994) *Biochemistry 33*, 12063−12071.
30. Putnam-Evans, C., and Bricker, T. M. (1992) *Biochemistry 31*, 11482−11488.
31. Putnam-Evans, C., Wu, J., Burnap, R., Whitmarsh, J., and Bricker, T. M. (1996) *Biochemistry 35*, 4046−4053.
32. Haag, E., Eaton-Rye, J. J., Renger, G., and Vermaas, W. F. J. (1993) *Biochemistry 32*, 4444−4454.
33. Eaton-Rye, J. J., and Vermaas, W. F. J. (1991) *Plant Mol. Biol. 17*, 1165−1177.
34. Putnam-Evans, C., and Bricker, T. M. (1994) *Biochemistry 33*, 10770−10776.
35. Putnam-Evans, C., and Bricker, T. M. (1997) *Plant Mol. Biol. 34*, 455−463.
36. Miller, A.-F., and Brudvig, G. W. (1990) *Biochemistry 29*, 1385−1392.

BI991326R