Mutation of Phe-363 in the Photosystem II Protein CP47 Impairs Photoautotrophic Growth, Alters the Chloride Requirement, and Prevents Photosynthesis in the Absence of either PSII-O or PSII-V in *Synechocystis* sp. PCC 6803†

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ABSTRACT: The deletion of the amino acids between Gly-351 and Thr-365 within the large, lumen-exposed, hydrophilic region (loop E) of the photosystem II (PSII) chlorophyll *a*-binding protein CP47 produced a strain of *Synechocystis* sp. PCC 6803 that failed to assemble stable PSII centers [Eaton-Rye, J. J., and Vermaas, W. F. J. (1991) *Plant Mol. Biol. 17*, 1165−1177]. The importance of two conserved Phe residues at positions 362 and 363 within this deletion has been investigated. The F363R strain had impaired photoautotrophic growth and an enhanced sensitivity to photoinactivation, demonstrating that Phe is required at position 363 for normal PSII function. In contrast, photoautotrophic growth in strains N361K and F362R was unaffected. Uniquely, among the mutant strains tested, F363R was unable to grow under chloride-limiting conditions, and this effect was reversed by replacing chloride with bromide. The removal of the manganese-stabilizing protein (PSII-O), the 12 kDa extrinsic protein (PSII-U), and cytochrome *c*-550 (PSII-V) was investigated in each mutant in vivo. In N361K and F362R, removal of PSII-V produced a more deleterious effect than the removal of PSII-O, but even so, all strains remained photoautotrophic. In contrast, the absence of PSII-V and PSII-O in F363R produced obligate photoheterotrophic strains. The removal of PSII-U increased the susceptibility of PSII to heat inactivation and further decreased the stability of PSII in F363R, demonstrating that PSII-U can contribute to the stabilization of mutations that have been introduced into CP47. The order of importance of the selective removal of the extrinsic proteins in strains carrying mutations in loop E of CP47 was found to be as follows: ΔPSII-V ≥ ΔPSII-O > ΔPSII-U.

In oxygen-evolving photoautotrophic organisms, the oxidation of water occurs within photosystem II (PSII[1]). The minimum number of protein subunits required to maintain a functional water-oxidizing complex (WOC) has not yet been established; however, the chlorophyll *a*-binding antenna proteins CP47 and CP43, together with the reaction center proteins, D1 and D2, and the α- and *β*-subunits of cytochrome *b*-559 are essential components of PSII core preparations, and additional low-molecular mass polypeptides may also be important. In vivo, however, the photosystem contains as many as 20 subunits, and this includes three hydrophilic proteins attached to the lumenal surface of the membrane that act as regulatory components (reviewed in refs *1* and *2*). In green plants, including the green algae, these extrinsic proteins, PSII-O, PSII-P, and PSII-Q, are encoded by the nuclear genes *psb*O, *psb*P, and *psb*Q, respectively. The PSII-O protein stabilizes manganese in the WOC, and PSII-P and PSII-Q are associated with calcium and chloride binding to the photosystem (reviewed in ref *3*). In non-green plants and cyanobacteria, PSII-P and PSII-Q are absent but two different extrinsic proteins, PSII-U and cytochrome *c*-550 (PSII-V), are present (*4*−*7*). It has been suggested that PSII-U and PSII-V stabilize the WOC during heat stress in *Synechococcus* sp. PCC 7002 (*8*, *9*), and a role of PSII-V in protecting the Mn cluster of the WOC from endogenous reductants has also been proposed (*10*, *11*).

The CP47 protein contains six hydrophobic α-helices which span the thylakoid membrane, and these are separated by five hydrophilic loop domains A−E. Both the N-terminus and C-terminus face the cytosol (or stroma in eukaryotes), while loops A, C, and E face the lumen (*12*, *13*). Loop E, between the fifth and sixth α-helices, contains about 200 amino acids and is protected from amino acid modification and digestion with proteases by the PSII-O protein which has also been shown to cross-link to this domain (*14*−*19*). In addition, the monoclonal antibody FAC2 recognizes an antigenic determinant within the hydrophilic loop following the removal of the extrinsic proteins and the strongly bound manganese pool associated with the WOC (*20*, *21*).

The introduction of short amino acid deletions has been used to map functional domains within loop E in the cyanobacterium *Synechocystis* sp. PCC 6803 (*22*, *23*). A deletion between Gly-351 and Thr-365 prevented the assembly of stable PSII centers and therefore blocked photoautotrophic growth (*22*). In contrast, an overlapping deletion

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[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; OD, optical density; WOC, water-oxidizing complex; PCC, Pasteur Culture Collection; PSII, photosystem II; TES, 2-{[tris(hydroxymethyl)methyl]amino}-1-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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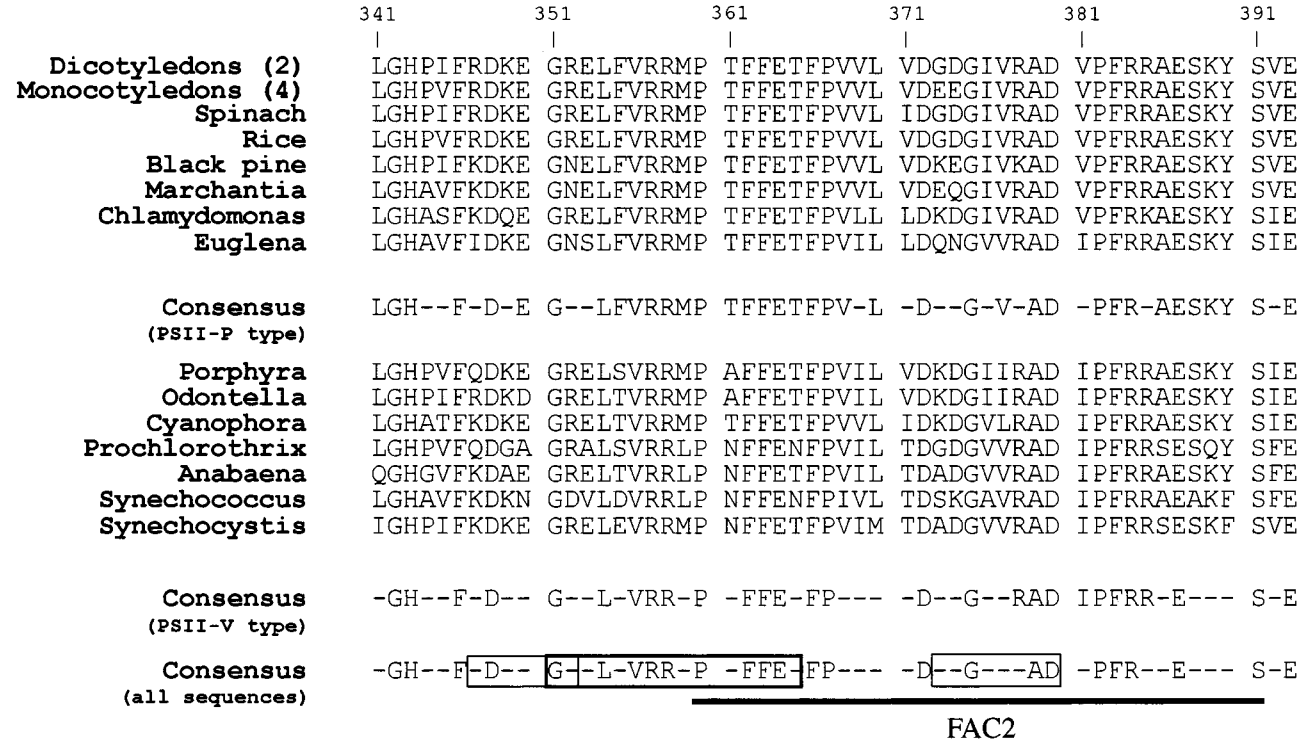

FIGURE 1: Diagram of the CP47 sequence of loop E between Leu-341 and Glu-393. The location of the deletions Δ(K347−R352) and Δ(A373−D380) are shown in boxes, and the deletion Δ(G351−T365) is shown in a box with bold lines. The epitope for monoclonal antibody FAC2 (*17*, *20*, *21*) is shown as a bold line underneath the consensus sequence between Pro-360 and Ser-391. The alignment of the CP47 sequences was constructed from the GenBank database using the GCG software package (*44*, *45*). The identical dicotyledons are *Oenothera hookeri* (accession number X55900) and *Nicotiana tabacum* (accession numbers Z00044 and S54304), and the identical monocotyledons are *Hordeum vulgare* (accession number X14107), *Secale cereale* (accession number X07672), *Triticum aestivum* (accession number X54749), and *Zea mays* (accession number X05422). The other species are *Spinacia oleracea* (accession numbers X02945 and X00471), *Oryza sativa* (accession number X15901), *Pinus thunbergiana* (accession number D17510), *Marchantia polymorpha* (accession numbers X04465 and Y00686), *Chlamydomonas reinhardtii* (accession number M84022), *Euglena gracilis* (accession numbers X15903 and S55899), *Porphyra purpurea* (accession number U38804), *Odontella sinensis* (accession number Z67753), *Cyanophora paradoxa* (accession number U30821), *Prochlorothrix hollandica* (accession number X59614), *Anabaena* sp. PCC 7120 (accession number X58847), *Synechococcus* sp. PCC 7942 (accession number Z14087), and *Synechocystis* sp. PCC 6803 (accession number M17109).

Δ(K347−R352) was photoautotrophic and contained levels of PSII close to that of the wild type, while the neighboring deletion Δ(A373−D380) could both assemble PSII and support photoautotrophic growth, although it was sensitive to photoinactivation (*23*). It has been suggested that the photoheterotrophic phenotype of Δ(G351−T365) is due to the large size of the deletion (*23*−*25*), but this is unlikely to be the sole reason because the introduction of a random amino acid sequence between Gly-351 and Thr-365 did not restore photoautotrophic growth, suggesting that this domain contains one or more essential amino acids (*11*).

A number of substitutions have been created for amino acids that are deleted in the Δ(G351−T365) strain. Putnam-Evans et al. (*24*, *25*) produced the mutant strain P360G and introduced other mutations at conserved charged residues, producing the additional strains RR357,358GG and E364Q, and the mutant E364G has also been produced (*11*). Each of these strains retained a phenotype that was similar to that of the wild type. Similarly, mutations at Thr-365, Gly-351, Glu-353, and Glu-355 produced little or no detrimental effect on PSII stability or function (*11*). This study has introduced substitutions at Asn-361, Phe-362, and Phe-363 which fall within the epitope for the monoclonal antibody FAC2 (Figure 1). Each of these uncharged amino acids has been replaced by basic residues, and each substitution has been studied, in vivo, in the presence or absence of PSII-O, PSII-U, and PSII-V. Our results demonstrate that Phe-363 is important in PSII assembly and/or stability with the mutant F363R, both retarding photoautotrophic growth and altering the chloride requirement for growth and water-splitting activity. The results also show that the removal of PSII-O or PSII-V in F363R prevents photoautotrophic growth, and while the removal of PSII-U produces a less severe phenotype, the stability of PSII in F363R:ΔPSII-U is further diminished when compared with the stability of the photosystem in F363R.

MATERIALS AND METHODS

Mutant Construction and Verification. Oligonucleotide-directed mutagenesis was used to construct mutant strains N361K, F362R, and F363R, as described by Eaton-Rye and Vermaas (*22*). The mutagenic oligonucleotide 5′-GTATGC-CTAAATTCTTTG-3′ was used to generate the Asn to Lys change at position 361; the Phe to Arg change at position 362 was generated using the oligonucleotide 5′-TGC-CTAACCGCTTTGAAACT-3′, and the F363R amino acid substitution was generated with the oligonucleotide 5′-CCTAACTTCCGTGAAACTTTC-3′. The bases that were changed have been underlined, and plasmids containing the *psb*B gene after mutagenesis were sequenced to confirm the presence of the intended substitutions. Following verification, the *psb*B fragment containing the desired mutation was excised and cloned into a plasmid that also led to restoration of a complete kanamycin-resistance cassette, thereby provid-

ing a method of selection (*22*). Restriction enzyme digests were used to test the resulting constructs isolated from single *Escherichia coli* clones prior to transformation of the photoheterotrophic *psb*B deletion strain of *Synechocystis* sp. PCC 6803 (*22*). Genomic DNA was isolated from the mutant strains, and the *psb*B gene was amplified by PCR and sequenced to confirm the presence of the introduced mutation as described by Morgan et al. (*11*).

The strains lacking PSII-O were created by transformation of the control and mutants with a pUC19 plasmid containing the *psb*O gene that had been interrupted by a spectinomycin-resistance cassette (*11*). For the creation of *psb*V deletions, a plasmid in which an intragenic 0.4 kb *psb*V fragment had been replaced by an erythromycin-resistance cassette was used to transform the control and mutant strains. This plasmid has been described by Shen et al. (*26*). To create the mutants which lacked the PSII-U protein, a pUC19 plasmid, containing the *psb*U gene that had been interrupted with a chloramphenicol-resistance cassette, was transformed into the control and mutant strains (*27*). Verification of the mutant strains lacking the extrinsic proteins was performed as described by Morgan et al. (*11*). A PCR was performed to show that the strains carrying the deletion of *psb*V were homozygous, and Western analysis was performed to confirm the absence of PSII-V. Southern and Western analyses were performed to confirm the interruption of *psb*O and *psb*U and the absence of PSII-O or PSII-U, respectively.

Growth Conditions. Control and mutant *Synechocystis* sp. PCC 6803 cultures were maintained on BG-11 plates in the presence of 5 mM glucose, 20 μM atrazine, and the appropriate antibiotics. When kanamycin, spectinomycin, and erythromycin were present, these were at a concentration of 25 μg/mL and chloramphenicol was used at a concentration of 20 μg/mL. The BG-11 solid medium was supplemented with 10 mM TES/NaOH (pH 8.2) and 0.3% sodium thiosulfate. Cultures were grown in liquid BG-11 media in the presence of antibiotics and were aerated with filtered air using an aquarium pump. Where appropriate, glucose was added at a final concentration of 5 mM. Cultures were maintained under constant illumination of 25 μE m^{-2} s^{-1} at 30 °C. Photoautotrophic growth curves were set up as described by Morgan et al. (*11*) with the addition of the appropriate antibiotic to each culture. For experiments performed under chloride-limiting conditions, $MnCl_2$ was replaced with $MnSO_4$ and $CaCl_2$ was replaced with $Ca(NO_3)_2$, and this reduced the chloride to a level that could not be detected by titration with sodium borohydride (*28*). Sodium bromide at 480 μM was added to the chloride-deficient BG-11 media for experiments performed in the presence of bromide.

Quantitation of Photosystem II. The variable chlorophyll fluorescence yield measurements were performed on an OCCAM Technologies kinetic fluorimeter to determine the relative concentrations of PSII reaction centers. Measurements were performed in the presence of 20 mM hydroxylamine and 40 μM diuron as described by Nixon and Diner (*29*). The relative amount of assembled PSII centers was also estimated on a chlorophyll basis with herbicide-binding assays employing [^{14}C]atrazine as described in ref *11*. The specific activity of the [^{14}C]atrazine was 18.6 mCi/mmol, and the chlorophyll concentration was 50 μg/mL. The samples were incubated for 30 min in the dark and were shaken every 10 min.

Oxygen Evolution. A Clark-type oxygen electrode maintained at a constant temperature of 30 °C was used to measure the rate of photosynthetic oxygen evolution. Measurements were taken at a chlorophyll concentration of 10 μg/mL in BG-11 containing 25 mM HEPES/NaOH (pH 7.5). Actinic light was provided by a Schott 1500 light source providing 6500 μE m^{-2} s^{-1} light passed through a 515 nm cutoff filter. The electron acceptors were 1.0 mM $K_3Fe(CN)_6$ and 0.2 mM 2,5-dimethyl-*p*-benzoquinone. The chlorophyll determinations for oxygen evolution and the other assays in this paper were performed according to the methods of MacKinney (*30*). For chloride-deficient measurements, BG-11 without chloride was prepared as described for the growth conditions.

Heat Inactivation Experiments. Cultures were grown and harvested as described above and washed twice in BG-11 containing 25 mM HEPES/NaOH (pH 7.5). Cells were then resuspended at a chlorophyll concentration of 30 μg/mL and incubated in the dark for 20 min in a 1 mL volume at various temperatures. Cells were then diluted to a chlorophyll concentration of 10 μg/mL at 30 °C, and the rate of oxygen evolution was measured after equilibration for 2 min.

Photoinactivation Experiments. *Synechocystis* sp. PCC 6803 control and mutant cultures were grown and harvested as described above and washed twice in BG-11 containing 25 mM HEPES/NaOH (pH 7.5). Cells were resuspended at a chlorophyll concentration of 10 μg/mL at 30 °C and preilluminated with 3800 μE m^{-2} s^{-1} white light. After the photoinactivating treatment, the rate of oxygen evolution was measured with 6500 μE m^{-2} s^{-1} light that had been passed through a 515 nm cutoff filter. Controls were also performed without the preillumination light on.

RESULTS

Verification of Mutant Strains. The location and sequence alignment of the amino acids that have been substituted in this study are shown in Figure 1, and the consensus sequences for those organisms that have PSII-P and PSII-Q and those that have PSII-V and PSII-U are presented. The location of the deletion in strain Δ(G351−T365) is also shown together with the overlapping and neighboring photoautotrophic mutants, Δ(K347−R352) and Δ(A373−D380). Figure 1 also illustrates that Asn-361, Phe-362, and Phe-363 are found at the beginning of the FAC2 epitope (*17*, *20*, *21*). To confirm the identity of the different mutants, the genomic DNA of N361K, F362R, and F363R, as well as that from the strains in which the extrinsic proteins were absent, was isolated, amplified, and sequenced for verification of the introduced mutation into the *psb*B gene. In addition, to verify the absence of PSII-O, PSII-U, and PSII-V in the corresponding mutant strains, PCR, Southern, and Western analyses were performed as described by Morgan et al. (*11*) (data not shown).

Characterization of Mutant Strains. The photoautotrophic growth curves for N361K, F362R, F363R, and the control strain are shown in Figure 2A. The control strain of *Synechocystis* sp. PCC 6803 contains a kanamycin-resistance cassette located downstream of the *psb*B gene and is identical to the wild type except for the antibiotic-resistant phenotype

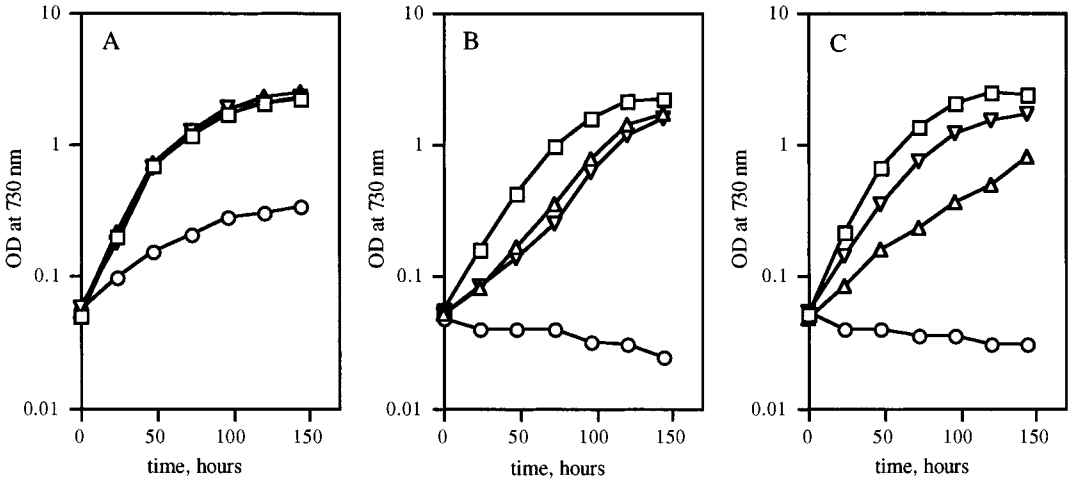

FIGURE 2: Photoautotrophic growth of *Synechocystis* sp. PCC 6803 strains as measured by the optical density at 730 nm in BG-11 media in the presence of the extrinsic proteins (A), in the absence of PSII-O (B), and in the absence of PSII-V (C): control (□), N361K (▽), F362R (△), and F363R (○).

Table 1: Characterization of *Synechocystis* sp. 6803 Strains in the Presence and Absence of the PSII-O, PSII-U, and PSII-V Proteins

strain	rate of oxygen[a] evolution	TFI (s)[b]	variable fluorescence yield[c]	PS II abundance determined by herbicide binding[d]
control	1.00	St[e]	1.00	1.00
ΔPSII-O	0.68	179	0.64	0.72
ΔPSII-U	0.96	187	0.51	1.00
ΔPSII-V	0.70	119	0.54	0.56
N361K	0.84	St[e]	0.73	0.79
ΔPSII-O	0.62	150	0.41	0.50
ΔPSII-U	0.83	189	0.61	0.85
ΔPSII-V	0.40	46	0.35	0.55
F362R	0.73	St[e]	0.73	0.76
ΔPSII-O	0.62	165	0.49	0.46
ΔPSII-U	0.81	175	0.58	0.65
ΔPSII-V	0.27	50	0.28	0.49
F363R	0.63	180	0.38	0.41
ΔPSII-O	0.19	25	0.10	ND[f]
ΔPSII-U	0.59	70	0.32	0.41
ΔPSII-V	0.00	0	0.13	ND[f]

[a] Normalized to control rate of 510 ± 40 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}. [b] Time to full inactivation (TFI) is the time for oxygen evolution to become completely inhibited during continuous actinic illumination. [c] Normalized to a variable fluorescence yield of 0.51 for the control. [d] The values for PSII abundance are normalized to a chlorophyll/PSII ratio of 560 and have been determined by [^{14}C]atrazine binding. [e] Stable rate of oxygen evolution. [f] No measurable [^{14}C]atrazine binding could be detected. The data are an average of three to five independent measurements and were reproducible within 15% of the average.

(*22*). The photoautotrophic doubling times for the control, N361K, and F362R strains were all found to be approximately 12 h; however, the strain F363R had an initial doubling time of 24 h and reached a plateau with an OD at 730 nm of approximately 0.5 after 100 h. The stationary phase for the control strain, N361K, and F362R occurred after approximately 125 h and had an OD at 730 nm of 2.5. An estimation of the relative levels of assembled PSII centers in these strains was obtained by measuring the variable chlorophyll fluorescence yield and is presented in Table 1. In the case of F363R, the relative level of PSII was reduced by 62% with respect to that observed for the control strain, while the relative levels of PSII in N361K and F362R were both reduced by 27%. Therefore, under the conditions employed, a reduction of approximately 27% in the amount of PSII present does not affect the photoautotrophic doubling time. To further characterize these strains, the rate of PSII oxygen evolution was measured and is also presented in Table 1. The mutant strains N361K and F362R were able to support stable oxygen evolution with N361K displaying 84% of the initial control rate and F362R showing 73%. F363R was able to support 63% of the initial rate of oxygen evolution observed for the control strain; however, this rate was not maintained and became fully inactivated in 180 s. Each of the above strains was then analyzed in the absence of either PSII-O, PSII-U, or PSII-V.

Upon removal of the extrinsic PSII-O protein, the photoautotrophic doubling time for the control, N361K, and F362R strains was extended to approximately 20, 29, and 25 h, respectively, and N361K and F362R exhibited a slight initial lag in their photoautotrophic growth (Figure 2B). However, F363R:ΔPSII-O was unable to support photoautotrophic growth. From the variable fluorescence yield data in Table 1, it can be seen that removal of the PSII-O protein resulted in fewer assembled PSII centers in all strains. The control:ΔPSII-O strain retained 64% of the control level with N361K:ΔPSII-O, F362R:ΔPSII-O, and F363R:ΔPSII-O only retaining 41, 49, and 10%, respectively. Table 1 shows that a decreased and less stable rate of oxygen evolution was also observed for all strains without the PSII-O protein where control:ΔPSII-O, N361K:ΔPSII-O, and F362R:ΔPSII-O supported between 62 and 68% of the initial control rate and became fully inactivated after 150−179 s of continuous illumination and F363R:ΔPSII-O displayed only 19% and was completely inactivated in 25 s.

Removal of the 12 kDa extrinsic protein, PSII-U, did not affect the photoautotrophic growth of the control and mutant strains (data not shown), and the initial rate of oxygen evolution was not impaired by the removal of PSII-U; however, a stable rate was not maintained at 6500 μE m^{-2} s^{-1}. In Table 1, the oxygen evolution rates for control:ΔPSII-U, N361K:ΔPSII-U, and F362R:ΔPSII-U reached full inactivation between 175 and 189 s with that for F363R:ΔPSII-U only taking 70 s to be completely inactivated. The variable fluorescence yield was also found to be significantly reduced in the ΔPSII-U strains. However, quantitation of PSII on a chlorophyll basis by measuring the level of diuron-replaceable atrazine binding in whole cells produced no

difference in PSII levels in strains lacking PSII-U when compared to the control level (Table 1). In contrast, the relative levels of PSII present, in the presence of all three extrinsic proteins or in the absence of PSII-O, are similar if measured by either variable fluorescence yield or level of herbicide binding. The agreement between these two methods for estimating the relative level of PSII has been seen previously (*31*). Therefore, removal of PSII-U introduces a significant amount of fluorescence quenching as well as a susceptibility to photoinactivation at high light levels. The origin of the fluorescence quenching has yet to be identified.

The PSII-V protein was also removed from the control and mutant strains. The removal of this extrinsic protein generally produced a phenotype that was similar to, or more severe than, that resulting from the removal of PSII-O. Photoautotrophic growth for control and N361K in the absence of PSII-V had a doubling time of 14 and 18 h, respectively, and the doubling time for F362R:ΔPSII-V was extended to 27 h. Photosynthetic oxygen evolution in control:ΔPSII-V exhibited a rate that was 70% of that observed for the control strain and was fully inactivated in 119 s as seen previously (*11*). The relative number of assembled PSII centers was also determined to be 54% of the control level when the variable fluorescence yield was measured, and a similar level was determined on a chlorophyll basis with the herbicide-binding assay. It can also be seen in Table 1 that the level of PSII in N361K:ΔPSII-V and F362R:ΔPSII-V remained at a level similar to that of control:ΔPSII-V when measured by herbicide binding; however, both the rate and stability of oxygen evolution decreased in both mutants, and the variable chlorophyll fluorescence yield was apparently quenched.

Strain N361K:ΔPSII-V produced 40% of the initial rate of oxygen evolution and 35% of the level of the variable fluorescence yield measured in the control or 48% of the level determined for both of these parameters in N361K. In the case of F362R:ΔPSII-V, the variable fluorescence yield was 28% of that found in the control and F362R:ΔPSII-V exhibited 27% of the initial control rate of oxygen evolution. The level of these parameters when compared to F362R was 37% for oxygen evolution and 38% of the fluorescence yield. In both N361K:ΔPSII-V and F362R:ΔPSII-V, the oxygen evolution activity was fully inactivated after 46 and 50 s, respectively. Strain F363R:ΔPSII-V was unable to support photoautotrophic growth, and no PSII centers could be detected by herbicide binding due to the accuracy of this assay when too few centers are present. However, at least 13% of the level of assembled PSII in the control strain could be detected by the fluorescence assay, but no oxygen evolution could be measured in this mutant.

Heat Inactivation of F363R and F363R:ΔPSII-U. To investigate if the deletion of PSII-U further destabilized PSII in F363R, the heat inactivation of oxygen evolution was assessed in both F363R and F363R:ΔPSII-U. From Figure 3, it can be seen that removal of the PSII-U protein from the control strain destabilized the PSII reaction center. A 50% inactivation of oxygen evolution was observed at approximately 49 °C in the control strain, but this was reduced to approximately 42 °C in control:ΔPSII-U. In the case of F363R, the temperature resulting in a 50% reduction in oxygen-evolving activity was approximately 41 °C, and this was reduced further to approximately 38 °C in F363R:ΔPSII-U.

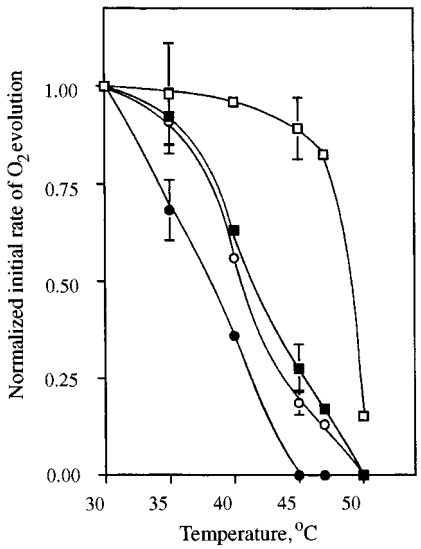

FIGURE 3: Heat inactivation of PSII oxygen evolution by the control (squares) and F363R (circles) strains in the presence (white symbols) or absence (black symbols) of the PSII-U protein. The cells were treated for 20 min at the designated temperature in darkness, and PSII activity was measured at 30 °C. Oxygen evolution rates were normalized to the initial rate measured at 30 °C following a 20 min pretreatment at 30 °C. The data are an average of three independent measurements. The standard error bars are indicated for the 35 and 45 °C points for each strain.

Photoinactivation of F363R. Since the observed rate of oxygen evolution for F363R was fully inactivated in 180 s, the sensitivity of this strain to photoinactivation was further investigated, and the results are shown in Figure 4. The effect of different preillumination treatments on the initial rate of oxygen evolution is shown in Figure 4A. As a control, both strains were subjected to a 15 min dark incubation period, and when assayed, the initial rate of oxygen evolution for the control remained at 95% of that observed when no pretreatment was given and the corresponding rate for F363R remained at 102%. In contrast, the initial rate of oxygen evolution for the control strain after the 15 min photoinactivating treatment with 3800 μE m^{-2} s^{-1} white light decreased to 54% of the initial rate, and only 33% of the initial rate remained in the F363R mutant after the treatment. In Figure 4B, the sensitivity to photoinactivation in F363R is seen to be more severe when the stability of the reaction was measured over approximately 3.5 min. In Figure 4B, the control strain was able to evolve oxygen at a relatively stable rate after a 15 min preillumination; however, the F363R strain was completely inactivated after less than 100 s of actinic illumination following the pretreatment.

Characterization of F363R under Chloride-Limiting Conditions. We have recently found that a strain carrying a deletion between Arg-385 and Val-392, which is within the FAC2 epitope (Figure 1), was significantly inhibited in chloride-limiting BG-11 medium (*11*). Since chloride is an essential cofactor for PSII activity (reviewed in ref *32*), we assessed the performance of N361K, F362R, and F363R under chloride-limiting conditions. Photoautotrophic growth in chloride-limiting media resulted in an increase in the doubling time for control and N361K to 20 h, while F362R exhibited a greater effect with the doubling time being extended to 46 h (Figure 5A,B).

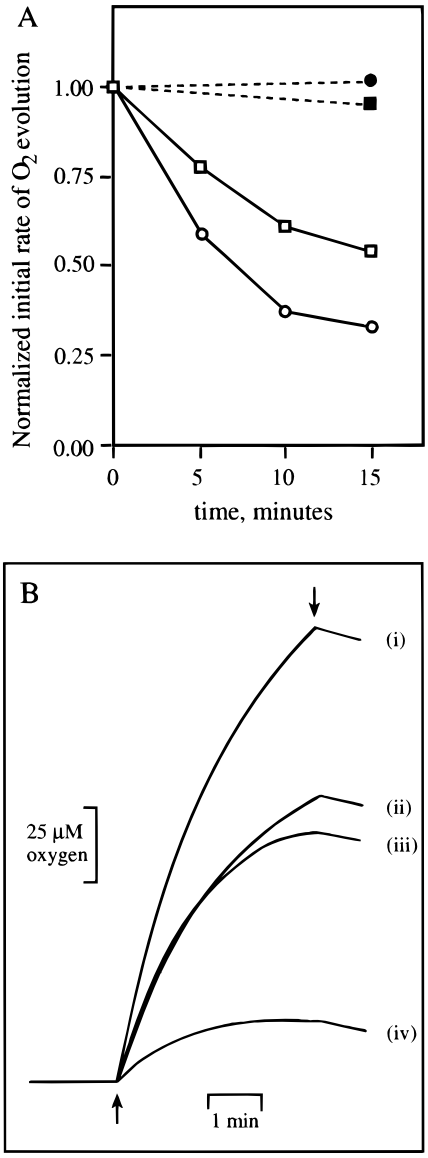

FIGURE 4: Photoinactivation of PSII oxygen evolution by the control (squares) and F363R (circles) strains. (A) Normalized initial rate of oxygen evolution after preillumination with white light at 3800 μE m^{-2} s^{-1} for the specified times. The black symbols correspond to data from control measurements taken after a 15 min dark incubation before the rate of oxygen evolution was measured. (B) Traces of oxygen concentration from cells of *Synechocystis* sp. PCC 6803 as determined with a Clark electrode and measured at 6500 μE m^{-2} s^{-1}. The arrows indicate when the light was turned on and off: (i) control trace with no preillumination, (ii) control trace after a 15 min preillumination with white light at 3800 μE m^{-2} s^{-1}, (iii) F363R trace with no preillumination, and (iv) F363R trace after a 15 min preillumination with white light at 3800 μE m^{-2} s^{-1}.

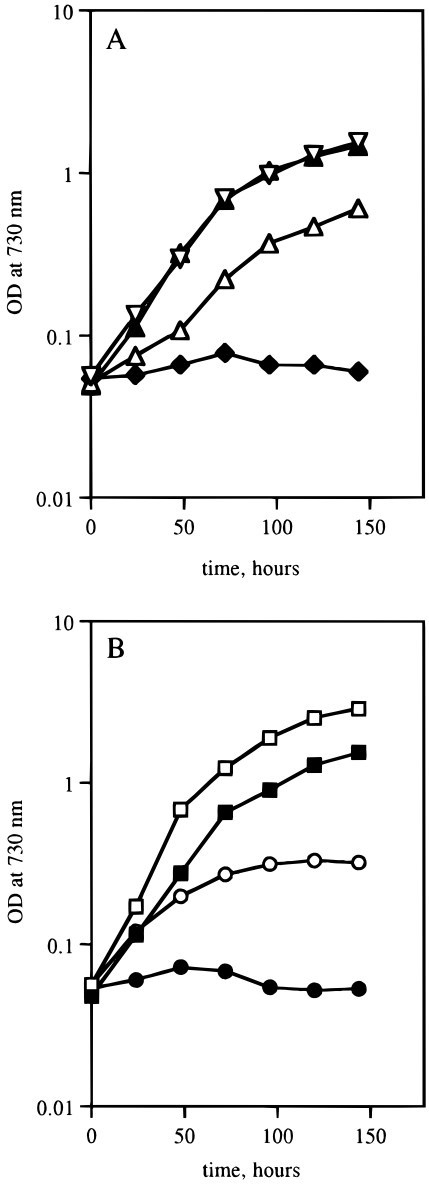

FIGURE 5: (A) Photoautotrophic growth of N361K (▽), F362R (△), E364G (▲), and FF362,363RR (◆). (B) Photoautotrophic growth of the control (squares) and F363R (circles) strains as measured by the optical density at 730 nm in chloride-limiting BG-11 media (black symbols) and in chloride-limiting media supplemented with 480 μM NaBr (white symbols).

The chloride-limited growth curves for two additional strains, E364G and FF362,363RR, are also shown in Figure 5A. The photoautotrophic doubling time for E364G was found to be 19 h. Similarly, the triple mutant G351L/E364Q/T365Q did not exhibit a chloride requirement (*11*). In contrast, FF362,363RR did not support photoautotrophic growth and the cells remained in a stationary phase. This phenotype was also observed for F363R (Figure 5B). These results indicated that a specific chloride dependency was introduced by the F363R substitution.

It is known that bromide can functionally replace chloride in supporting oxygen evolution in higher-plant preparations (*33*, *34*), and bromide has also been shown to functionally replace chloride in cyanobacteria (*35*−*37*). From a comparison of Figures 5B and 2A, it can be seen that addition of 480 μM NaBr to chloride-deficient media restored the photoautotrophic growth in both the control strain and F363R to the level observed in normal BG-11. Oxygen evolution was also assessed after both the control and the F363R strain were grown in the presence of 5 mM glucose and in the presence or absence of chloride or bromide. From Table 2, it can be seen that the different growth conditions did not have a major impact on the ability of the control strain to evolve oxygen when measured in the presence of chloride; however, when the strains were grown without chloride or bromide, a stable rate was not observed and the PSII activity was completely inactivated in 195 s. When the strains were grown in either the presence or absence of chloride and the rate was measured in the absence of chloride, the resulting

Table 2: Characterization of the Control Strain and F363R Grown in Normal BG-11, Chloride-Limiting BG-11, and BG-11 with Bromide Substituted for Chloride

strain	growth condition	variable fluorescence yield	rate of oxygen evolution		TFI (s)[a]	
			with Cl	without Cl	with Cl	without Cl
control	with Cl[b]	1.00[c]	1.00[c]	0.83	St[d]	218
	without Cl	0.85	0.85	0.83	195	165
	with Br	1.27	1.06	0.79	St[d]	188
F363R	with Cl[b]	0.38	0.63	0.50	180	127
	without Cl	0.34	0.31	0.19	79	44
	with Br	0.39	0.60	0.40	180	83

[a] Time to full inactivation (TFI) is the time for oxygen evolution to become completely inhibited during continuous actinic illumination when assayed in the presence (with Cl) or absence (without Cl) of chloride. [b] The data for the normal BG-11 (with Cl) are the same as those shown in Table 1 with the exception that the oxygen evolution measurements in the absence of chloride are included above. [c] The variable fluorescence yield and rate of oxygen evolution for the control are the same as in Table 1. [d] Stable rate of oxygen evolution. The data are an average of three to five independent measurements and were reproducible within 15% of the average.

rate of oxygen evolution for the control was both reduced and susceptible to photoinactivation. The mutant strain F363R, even though unable to support photoautotrophic growth in the absence of chloride, was still capable of evolving oxygen when it was grown in the presence of 5 mM glucose in chloride-limiting media. However, this rate was fully inactivated in 79 s, but this was extended to 180 s when the strain was grown in the presence of chloride or bromide (Table 2). The observed rate of oxygen evolution for F363R was 36% of that observed for the control when it was grown under chloride-limiting conditions and assayed in the presence of chloride and 23% of the control rate when measured in the absence of chloride. However, when F363R was grown without chloride, but in the presence of bromide, oxygen was evolved at a rate similar to that determined for F363R cells grown in the presence of chloride. It was also found that when measured in the absence of chloride, the oxygen evolution rate in F363R was more susceptible to photoinactivation than that in the control strain. Interestingly, the different variations in growth conditions did not eliminate the ability of either the control or F363R to assemble PSII with both the strains possessing more than 80% of the level of PSII present when grown in chloride-sufficient BG-11.

We also note that in Table 2 the variable fluorescence yield in the presence of bromide, particularly in the control strain, was elevated when compared to that measured in normal BG-11. This result arises from an apparent quenching of F_o (the initial fluorescence yield measured in the dark-adapted sample) (see ref *38*, and references therein). The origin of this phenomenon is not known.

DISCUSSION

Residue Phe-363 Has Been Identified as a Critical Amino Acid between Gly-351 and Thr-365. The deletion strain Δ(G351−T365) does not assemble stable PSII reaction centers and therefore does not support photoautotrophic growth (*22*), and it has been suggested that this occurs as a result of the comparatively large size of the amino acid deletion (*23*−*25*). Moreover, we have recently created mutants L354K, V356Y, and M359D, and each of these strains grew photoautotrophically at a rate that was similar to the control (*39*); in addition, mutations at all the other positions between Gly-351 and Thr-365, except Asn-360, Phe-362, and Phe-363, did not appear to disrupt PSII function to any significant degree (*11*, *23*−*25*).

The results presented here for F363R are therefore striking as this is the first example of a conserved hydrophobic amino acid in loop E of CP47 playing a critical role in PSII assembly or function. The uniqueness of Phe-363 is highlighted by the fact that the introduction of a positive charge in strain N361K had only a minor effect when compared to the control, while the creation of the double mutant FF362,363RR produced a phenotype that was indistinguishable from F363R (*39*).

Removal of the Extrinsic Proteins. Previous results had established that the removal of PSII-V in E364G:ΔPSII-V and E364Q:ΔPSII-V prevented photoautotrophic growth while the removal of PSII-O in strain G351L/E364Q/T365Q:ΔPSII-O destabilized PSII and extended the photoautotrophic doubling time when compared to that of the control:ΔPSII-O strain (*11*). In contrast, the removal of either of these extrinsic proteins in F363R:ΔPSII-O or F363R:ΔPSII-V resulted in a loss of photoautotrophic growth, thereby demonstrating that F363R is more sensitive to the removal of PSII-O than substitutions at Glu-364. Also, in the control and the N361K and F362R strains, a more deleterious effect was observed upon the removal of PSII-V than upon the removal of PSII-O with generally fewer centers assembled, and the centers that were present exhibited an increased sensitivity to photoinactivation. This observation is in agreement with our previous report where the removal of PSII-V, in vivo, also produced severe phenotypes in several mutants with deletions or substitutions between Gly-351 and Thr-436 of loop E (*11*). In addition, an earlier report had also noted that the removal of PSII-V in the wild type destabilized PSII to a greater extent than the removal of PSII-O (*26*).

In this study, we have also been able to evaluate the effect of removing the PSII-U protein. Almost no impact was evident on photoautotrophic growth or the initial rate of oxygen evolution in the ΔPSII-U strains, but even in the control:ΔPSII-U strain, the observed rate of oxygen evolution was found to be unstable. The removal of PSII-U from the wild type has already been suggested to alter the normal advancement of the S-states of the WOC, and both the susceptibility to photoinactivation and the fluorescence quenching observed in the absence of PSII-U are presumably related to this phenomenon (*10*, *27*, *40*). Since it has also been reported that PSII-U plays a role in the heat stability of PSII in *Synechoccocus* sp. PCC 7002 (*9*), we investigated the effect of PSII-U on the heat stability of our CP47 mutants. In the case of *Synechococcus* sp. PCC 7002, the PSII-U protein was shown to stabilize the PSII complex against heat inactivation when detergent-treated thylakoid membranes were reconstituted with PSII-U in the absence of PSII-V. In our experiments with *Synechocystis* sp. PCC 6803, the *psb*U gene was interrupted with a gene conferring resistance to chloramphenicol and the effect of the removal of PSII-U was investigated in vivo in the presence of PSII-V. Under our conditions, we also observed a definite susceptibility to heat inactivation following the removal of PSII-U in the control:ΔPSII-U strain, and we observed a clear decrease in the

stability of PSII in F363R:ΔPSII-U compared to that of F363R, thus supporting a role of PSII-U in stabilizing PSII in the perturbed photosystem of F363R.

Chloride Requirement in F363R. Chloride is an essential cofactor of the WOC (reviewed in refs *32* and *41*), and current models assign chloride as an integral component of the active site (*35*, *42*). However, the exact location of chloride and its precise role have yet to be experimentally verified. Nevertheless, the identification of mutations in loop E of CP47 that alter chloride binding, thereby resulting in impaired PSII activity, further suggests that loop E is in close proximity to the WOC (*36*, *37*, *43*).

A wide range of mutations produce an altered chloride requirement. These include substitutions at basic residues Arg-448 and Lys-321 (*36*, *37*), the two deletion mutants Δ(R384−V392) and Δ(G429−T436) (*11*), and a number of strains obtained through combinatorial mutagenesis which contain a series of mutations between Asp-440 and Pro-447 (*43*). However, Phe-363 is again unique in being the first example of a hydrophobic amino acid that, when changed, introduces a chloride effect. Several possible explanations for why an altered chloride requirement could give rise to the observed phenotypes have been discussed by Putnam-Evans and Bricker (*36*, *37*) and Tichy and Vermaas (*43*).

Conclusions. The mutation of Phe-363 to Arg resulted in a phenotype with impaired photoautotrophic growth and an enhanced sensitivity to photoinactivation, thereby demonstrating that a conserved uncharged amino acid is required at this position for normal PSII performance. Although speculative, the resulting chloride requirement would be consistent with the perturbation in the photosystem of F363R altering chloride binding at the active site of the WOC. All the amino acids between Gly-351 and Thr-365 have now been changed by oligonucleotide-directed mutagenesis (*11*, *23*−*25*, *39*), but only F363R produced a significant mutant phenotype; therefore, the absence of Phe-363 in Δ(G351−T365) may be an important factor contributing to the inability of this strain to assemble PSII. In N361K and F362R, removal of PSII-V produced a more deleterious effect than the removal of PSII-O, although all strains remained photoautotrophic. In contrast, both F363R:ΔPSII-O and F363R:ΔPSII-V were obligate photoheterotrophic strains. The removal of PSII-U in vivo increased the susceptibility of PSII to heat inactivation, and the removal of PSII-U in F363R was found to further destabilize PSII, therefore showing that the association of PSII-U with the photosystem can contribute to the stabilization of mutations that have been introduced into CP47. These results indicate that the order of importance of the selective removal of the extrinsic proteins in strains carrying mutations in loop E of CP47 is as follows: ΔPSII-V ≥ ΔPSII-O > ΔPSII-U.

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